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Barbara Anne Murphy

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ABSTRACT OF DISSERTATION

Barbara Anne Murphy

The Graduate School

University of Kentucky

2007

INVESTIGATIONS OF CIRCADIAN REGULATION AND IMMUNE-CIRCADIAN  
INTERACTION IN THE HORSE

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy  
in the College of Agriculture  
at the University of Kentucky

By

Barbara Anne Murphy

Lexington, Kentucky

Director: Dr. Barry Fitzgerald, Associate Professor of Veterinary Science

Lexington, Kentucky

2007

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## ABSTRACT OF DISSERTATION

### INVESTIGATIONS OF CIRCADIAN REGULATION AND IMMUNE-CIRCADIAN INTERACTION IN THE HORSE

The circadian system provides animals with a means to adapt internal physiology to the constantly changing environmental stimuli that exists on a rotating planet. Light information is translated into molecular timing mechanisms within individual pacemaker cells of the mammalian hypothalamic suprachiasmatic nucleus (SCN) via transcriptional-translational feedback loops. Humoral and neural outputs from this 'master' clock result in circadian rhythms of physiology and behavior. The hierarchy of the circadian system involves SCN synchronization of cellular clocks within peripheral tissues so that differential transcriptional profiles in individual organs reflect their specific function. The first step to investigating equine circadian regulation was to identify and isolate the core components of the molecular clock in the horse. Successful isolation and sequencing of equine *Bmal1*, *Per2*, *Cry1* and *Clock* cDNAs revealed high sequence homology with their human counterparts. Real Time RT-PCR assays were subsequently designed to quantitatively assess clock gene expression in equine peripheral tissues. Synchronization of equine fibroblasts revealed temporal profiles of clock gene expression identical to those of the SCN and peripheral tissues of other species. However, while clock gene expression varies over time in equine adipose tissue, there was no observable oscillation of clock gene transcripts in equine blood. Spurred by recent reports of immune-circadian interactions, this novel finding prompted an investigation of clock gene expression in equine blood during a systemic inflammatory response. The results demonstrated that acute inflammation upregulates *Per2* and *Bmal1* in equine blood. Subsequent experiments identified neutrophils as the source of this upregulation and highlighted exciting new immune-circadian interplay during an innate immune response. Finally, the effect of a 6-h phase advance of the light/dark cycle, mimicking an easterly transmeridian journey, on circadian melatonin and core body temperature rhythms was investigated. In contrast to the gradual adaptation observed in other species, these markers of equine circadian phase adapt immediately to a time zone transition. Combined, the results of these experiments highlight important interspecies differences in circadian regulation with practical implications regarding the potential impact of jet lag on equine athletes. Furthermore, the results underline the relevance of chronobiological investigation in a large mammalian species such as the horse.

KEYWORDS: Equine, Circadian Rhythm, Clock Gene, Inflammation, Jet Lag

Barbara Anne Murphy

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August 28<sup>th</sup>, 2007

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INVESTIGATIONS OF CIRCADIAN REGULATION AND IMMUNE-CIRCADIAN  
INTERACTION IN THE HORSE

By

Barbara Anne Murphy

Dr. Barry P. Fitzgerald

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Director of Dissertation

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August 28<sup>th</sup>, 2007

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DISSERTATION

Barbara Anne Murphy

The Graduate School  
University of Kentucky

2007



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## LIST OF ABBREVIATIONS

ANOVA .....	Analysis of Variance
BAC .....	Bacterial Artificial Chromosome
BCS .....	Body Condition Score
BMAL1 .....	Brain-Muscle-Arnt-like protein 1
CRY1 .....	Cryptochrome 1
CT .....	Circadian Time
ECA .....	Equus Caballus Autosome
FISH .....	Fluorescence In Situ Hybridization
GUS .....	Beta-Glucuronidase
HSA .....	Homo Sapien Autosome
LD12:12 .....	12 h Light/12 h Dark photocycle
LPS .....	Lipopolysaccharide
NCBI .....	National Center for Biotechnology Information
NK .....	Natural Killer
NSAID .....	Non-Steroidal Anti-Inflammatory Drug
ORF .....	Open Reading Frame
PBMC .....	Peripheral Blood Mononuclear Cell
PBZ .....	Phenylbutazone
PER2 .....	Period 2
PGE2 .....	Prostaglandin E2
RIA .....	Radioimmunoassay
RT-PCR .....	Reverse-Transcription- Polymerase Chain Reaction
SCN .....	Suprachiasmatic Nucleus
SEM .....	Standard Error of the Mean
TLR .....	Toll-like Receptor
TNF $\alpha$ .....	Tumor Necrosis Factor $\alpha$
UTR .....	Untranslated Region
ZT .....	Zeitgeber Time



## LIST OF FILES

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## CHAPTER 1 – Literature Review

### 1.1 Adaptation to Life on a Rotating Planet

Long before the dawn of chronobiology as a field of science relating to the study of cyclic phenomena in living organisms, there was evidence to suggest that our earliest ancestors had a keen interest in monitoring the passage of time. The existence of monumental megalithic stone circles and burial tombs, designed by primordial architects to catch and direct the rays of the solstice sun, demonstrate an intimate knowledge and understanding of the daily and seasonal changes in the solar passage in early cultures (Figure 1.1). However, it was not until as recently as the last half century that the complexities of biological timing processes within organisms have begun to be unraveled. Just as the shadow cast by a sundial may signify the hour of a scheduled human activity, endogenous biological clocks play a vital role in the adaptation of an organism's physiology to environmental time cues. The common denominator between both living and non-living clocks is the role played by light.



**Figure 1.1** Drombeg Stone Circle, c. 150 BC, Co. Cork. The stones were arranged so that when standing between the two tallest 'portal' stones the sun sets over the short, flat stone on the winter solstice.

The presence of the sun and the continuous rotation of our planet around its own axis results in the ever changing cycles of light and dark. For this reason, cellular clock mechanisms have evolved that are sensitive to light and provide organisms with the ability to anticipate periods of activity and in doing so, optimize survival. Clock mechanisms have evolved from single-cell algae and fungi that are directly exposed and responsive to sunlight (Hastings and Sweeney 1960; Lee *et al.* 2000; Sommer *et al.* 1989), to higher order organisms in which light can penetrate clock containing cells in many parts of the body (Plautz *et al.* 1997). The strength of the evolutionary pressure was such that in fish, such as zebrafish, light can still directly penetrate and affect clock mechanisms of internal organs (Delaunay *et al.* 2000; Whitmore *et al.* 2000). As the complexity of the nervous system increased, centralization of the light receptive elements evolved such that the circadian clock containing cells became concentrated in the light-transducing parts of the nervous system (Buijs *et al.* 2003). In birds, light receptive cells are present only in the retina and pineal gland (Menaker 1971) where light can still directly penetrate the skull (Foster *et al.* 1985; Menaker and Underwood 1976), while in mammals transduction of the light signal to the central nervous system only occurs via glutamate secretion (Ding *et al.* 1997) from retinal terminals of the retino-hypothalamic tract (RHT) (Moore and Lenn 1972; Morin 1994). Proteins such as cryptochromes, which are conserved from plants to humans (Hsu *et al.* 1996), played an important role in the evolution of the mammalian light-transducing pathway. While initially essential for light reception in plants, their function shifted to a role in biological timing in mammals, where they are essential components of the molecular clockwork mechanism (van der Horst *et al.* 1999).

The first intellectual postulation that the response of plants and animals to environmental cycles was more than a passive exogenous reaction, and instead reflected an innate ability for internal timing, was introduced to an academic setting by Jean Jacques d'Ortous deMairan in 1729. His observation that leaf movements of light-sensitive plants continued in constant darkness set the stage for the beginning of a new field of science (DeMairan, 1729; cited by DeCoursey, 2004). However, it was not for another century that researchers attributed rhythmic behavioral patterns to

internal timing mechanisms. One of the pioneers of research in circadian rhythms was Erwin Bünning, a plant pathologist who was the first to propose the hypothesis that circadian rhythms have adaptive value for organisms by demonstrating that photoperiodic time measurement is controlled by an internal mechanism (Bünning and Moser 1969). Interest in biological timing soon spread, spurred on by the discovery that rhythmical bioluminescent flashing of the unicellular algae *Gonyaulax* persisted under conditions void of external environmental stimuli (Hastings and Sweeney 1960). In addition, the coining of the phrase “biological clock” to describe the physiological entity responsible for navigation in migratory birds (Kramer, 1952; cited by DeCoursey, 2004) was another pivotal point for the emerging field of chronobiology. Two scientists, Colin S. Pittendrigh, an investigator of circadian organization in *Drosophila* and Jürgen Aschoff, whose research focused on locomotor activity rhythms in mice, are considered the founders of modern biological rhythm research and are credited with developing the unifying concepts and key principles of biological clocks (Daan, 2001; Daan et al., 1996). It was not until one of the first international symposia on biological clocks in 1960 and a lecture given by Colin Pittendrigh entitled “Circadian Rhythms and the Circadian Organisation of Living Systems” that the principles of circadian biology began to be accepted as applicable to human societal and medical concerns (DeCoursey, 2004).

*“Suddenly, and on a world scale, endogenous timing was seen by scientists and lay people alike as a cardinal feature of living organisms on a rhythmically revolving planet. Biological clocks were core features of life. ”*

Excerpt from DeCoursey (2004)

As with any specialized scientific field, chronobiology is associated with its own set of terms and definitions. The word circadian, originating from the Latin *circa* (around) and *dies* (a day), refers to the approximate 24-h period length of free-running rhythms in the absence of environmental stimuli. Environmental cues such as light are required to entrain circadian rhythms to the natural cycle on a daily basis (Pittendrigh and Minis, 1964). They are referred to as entraining signals, as they

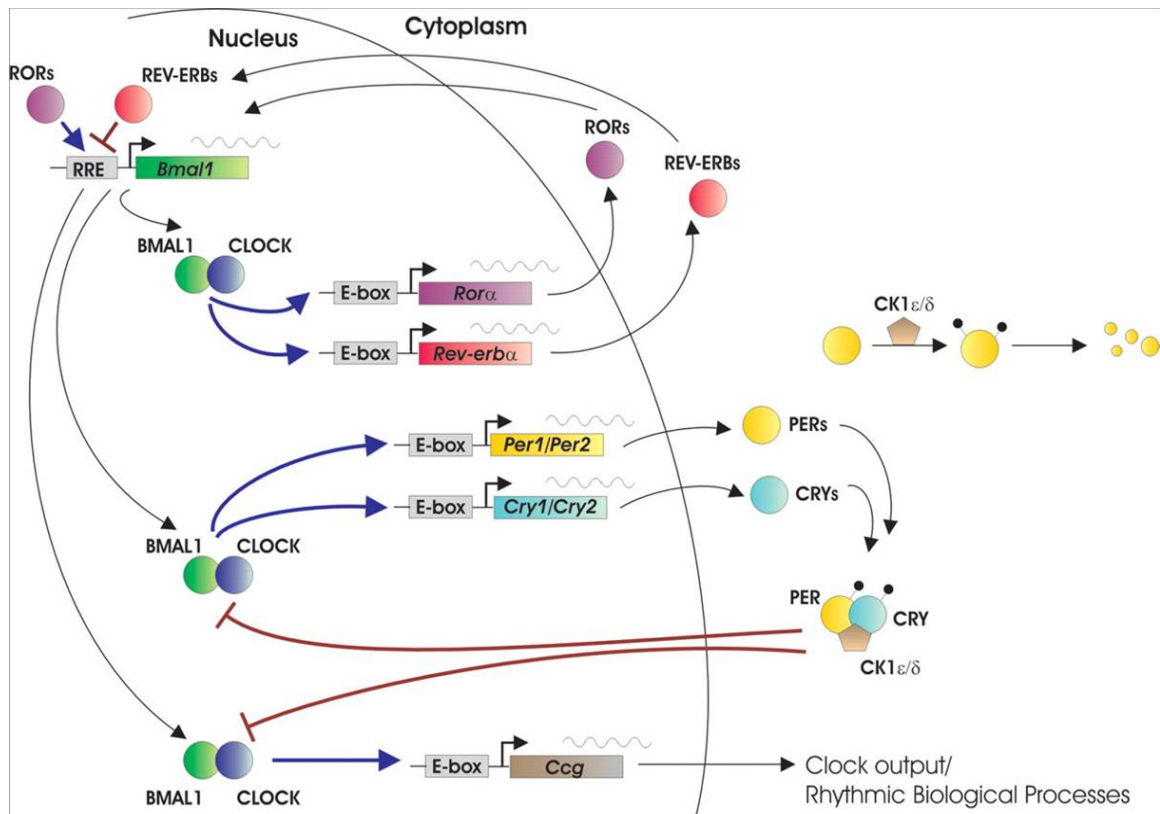
entrain, or ‘lock onto’ the driving oscillation of the environment. Entraining signals can also be referred to as zeitgebers, which literally translates to ‘time giver’, of which light is the primary time cue (Aschoff, 1965). A circadian rhythm is defined as an endogenous rhythm with a period length close to 24 h, that persists under constant conditions, is unaffected by changes in temperature and can be entrained by external time cues (Pittendrigh, 1960; Pittendrigh, 1993). The circadian system is made up of three component parts consisting of 1), an input or stimulus from the external environment necessary to synchronize 2), the central clock or pacemaker that drives 3), an output in the form of gene expression changes that influence physiological function and behavior. Outputs from the circadian clock are often termed overt rhythms as they are the physiological and/or behavioral endpoints that act as indirect markers of the internal clock.

The circadian rhythm of melatonin secretion from the pineal gland is one of the most stable outputs from the circadian clock (Benloucif *et al.* 2005) and represents one of the foremost mammalian adaptations to life on a rotating planet. Photoperiodic information travels from the retina to the seat of the mammalian pacemaker in the suprachiasmatic nucleus (SCN) (Moore and Eichler 1972; Stephan and Zucker 1972) of the hypothalamus. The molecular interplay of clock gene products within the SCN (the subject of the next section) controls the circadian output to the pineal gland (Perreau-Lenz *et al.* 2004) and dictates the timing of melatonin production and release (Bartness *et al.* 1993). Melatonin is synthesized and secreted solely during the dark period of the light/dark cycle and faithfully represents the duration of darkness, mirroring the seasonal changes in the length of day and night. Thus, this neuroendocrine pathway acts as a signal from the clock to the body conveying seasonal timing information to organs involved in reproduction in seasonal breeding animals (Bartness and Goldman 1989; Morgan and Mercer 1994). In this way, a circadian temporal component also ensures that physiological changes remain synchronized with the appropriate time of year.

## 1.2. Mechanisms of the Mammalian Molecular Clock

The mammalian master circadian clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus and regulates diverse physiological processes such as blood pressure, heart rate (Arraj and Lemmer 2006), sleep-wake cycles (Aston-Jones *et al.* 2001), hormone secretion (Weibel and Brandenberger 2002), metabolism (Kita *et al.* 2002) and body temperature (Moore and Danchenko 2002). The SCN consists of approximately 10,000 neurones located above the optic chiasma and about 3 cm behind the human eye (Hastings 1997). Photoc information transmitted via the RHT stimulates cell autonomous molecular clockwork mechanisms within each SCN neuron (Welsh *et al.* 2004). Core clock components have been defined as genes whose protein products are necessary for the generation and regulation of circadian rhythms within individual cells (Takahashi 2004). The molecular clock consists of gene-protein-gene feedback loops whereby the protein has a negative feedback effect on its own transcription and stimulates the transcription of other clock genes (Reppert and Weaver 2001). The core components can be assembled into a diagram of multiple interconnecting loops that involve complex transcriptional feedback circuits (Figure 1.2). The primary feedback loop consists of three *Period* genes (*Per1*, *Per2* and *Per3*), two cryptochrome genes (*Cry1* and *Cry2*), a *Clock* gene and the gene encoding brain-muscle-Arnt-like protein 1 (*Bmal1*) (Dunlap 1999). With the exception of *Clock*, which is constitutively expressed, all transcripts oscillate within the SCN, with *Per* and *Cry* transcripts peaking at roughly midday and those of *Bmal1* peaking at around midnight (Morse and Sassone-Corsi 2002). *Bmal1* transcription provides the positive driving force. CLOCK and BMAL1, both members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, form heterodimers that bind to E-box enhancer motifs upstream of the *Cry* (cryptochrome) and *Per* genes to initiate their transcription (Bunger *et al.* 2000;Gekakis *et al.* 1998;Kume *et al.* 1999;Zheng *et al.* 2001). PER and CRY proteins form complexes that translocate back into the nucleus and interfere with CLOCK-BMAL1 DNA binding to repress their own transcription (Kume *et al.* 1999;Lee *et al.* 2001;Okamura *et al.* 1999;Sato *et al.* 2006;Shearman *et al.* 2000). Simultaneously, each cycle of

BMAL1-CLOCK accumulation results in the cyclic transcriptional activation of an array of clock controlled genes (ccgs) that contribute to rhythmical biological processes outside of the clockwork mechanism. Recent studies have identified a second feedback loop induced by CLOCK-BMAL1 heterodimers comprising the opposing activities of the retinoic acid-related orphan nuclear receptors, *Rora* and *Rev-erba* (Preitner *et al.* 2002;Sato *et al.* 2004;Triqueneaux *et al.* 2004). In a competition to bind retinoic acid-related orphan receptor response elements (ROREs) present on the *Bmal1* promoter, members of the ROR $\alpha$  family activate transcription (Akashi and Takumi 2005;Guillaumond *et al.* 2005;Sato *et al.* 2006) whereas REV-ERBs repress the transcription process (Guillaumond *et al.* 2005;Preitner *et al.* 2002). In addition, post-translational modifications such as phosphorylation, particularly by members of the *casein kinase* (CK) family of enzymes, affect the stability and nuclear translocation of the core clock genes and contribute significantly to the time delays necessary for a 24 h clock (Akashi *et al.* 2002;Eide *et al.* 2002;Eide *et al.* 2005;Toh *et al.* 2001). The combined effect of both auto-regulatory feedback loops ensures perpetuation of the self-sustaining nature of the molecular clock. The high degree of complexity of the molecular clockwork continues to be revealed with recent demonstrations of crucial roles for new genes in maintaining the feedback loops that determine period length of circadian rhythms (Godinho *et al.* 2007;Honma *et al.* 2002;Siepka *et al.* 2007).



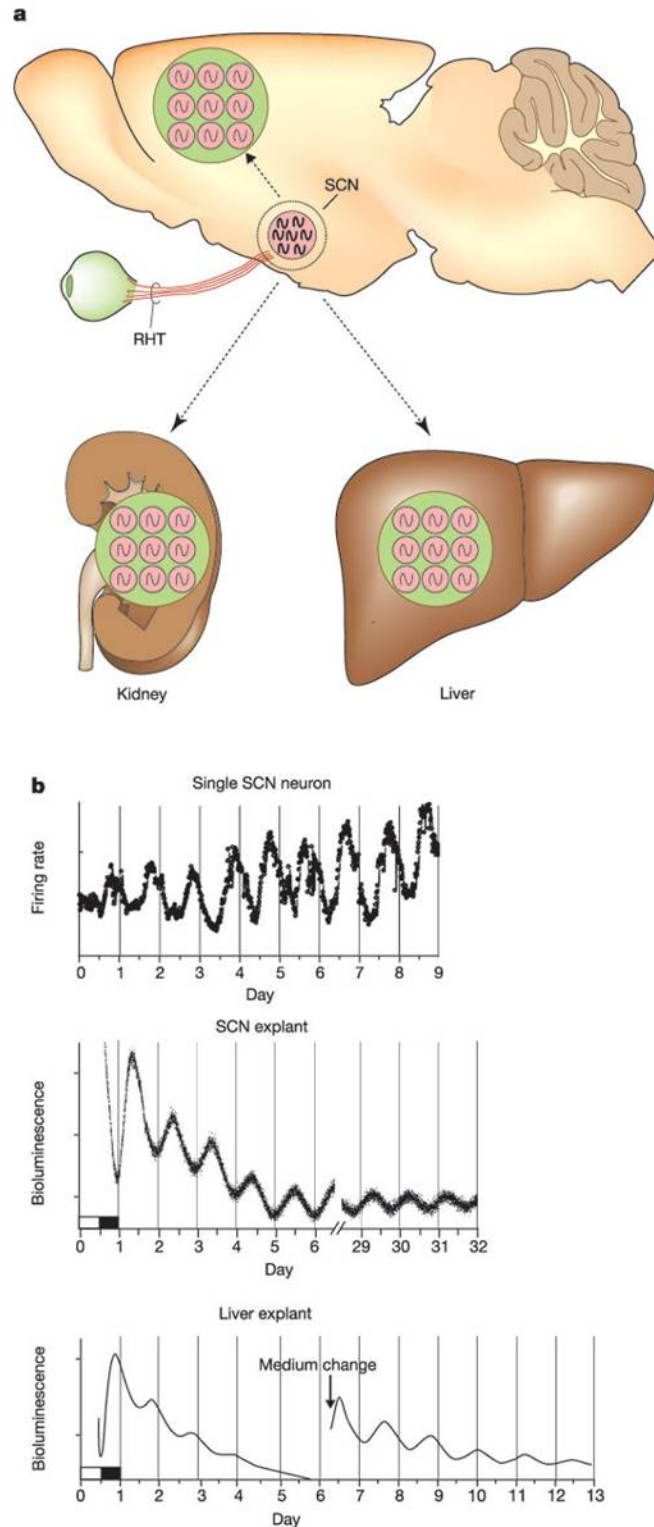
**Figure 1.2** The mammalian circadian clock consists of a network of transcriptional-translational feedback loops. From Ko, C. H. and Takahashi, J. S. Molecular components of the mammalian circadian clock. *Hum Mol Genet*, 2006, 15 Spec No 2: R271-7, by permission of Oxford University Press.

### 1.3. Hierarchy within the Circadian System

The mammalian circadian system is organized as a hierarchy of oscillators. The SCN resides at the top of this hierarchy and is responsible for coordinating independent peripheral oscillators in the same manner as a conductor might conduct an orchestra, such that overall rhythmic physiological harmony is achieved. Recent advances in the field of chronobiology have demonstrated that the molecular clock functions in almost all tissues and that possibly every cell in a given tissue contains an autonomous clock (Nagoshi *et al.* 2004; Welsh *et al.* 2004; Yoo *et al.* 2004).



Importantly, the molecular make-up and temporal expression relationships of clock gene mRNA rhythms within peripheral oscillators are identical to that of the SCN (Andersson *et al.* 2005; Muhlbauer *et al.* 2004; Oishi *et al.* 1998b; Yagita *et al.* 2001). A recent exception to this rule is *Clock*, whose expression has been shown to be rhythmic in some tissues outside of the SCN (Lowrey and Takahashi 2004). A master-slave relationship between SCN neurons and peripheral cells was first suggested following the observation of a 3-9 hour delay in the phase of clock gene oscillations between the SCN and the periphery (Zylka *et al.* 1998). Further discoveries revealed that while individual SCN neurons are capable of sustaining 24-h rhythms for weeks in culture (Welsh *et al.* 1995), rhythmical clock gene expression from explanted peripheral tissues quickly dampen in the absence of resetting stimuli from the SCN (Yamazaki *et al.* 2000) ( Figure 1.3). The ability of the SCN to reset rhythmical clock gene expression in cells of peripheral tissues has been demonstrated both *in vivo* (Ralph *et al.* 1990; Sujino *et al.* 2003) and *in vitro* (Allen *et al.* 2001), solidifying its position at the top of the circadian hierarchy. However, the perception that the SCN was responsible for driving rhythms in peripheral oscillators changed following the creation of a PER2::LUCIFERASE (PER2::LUC) fusion protein and the finding that isolated peripheral clocks could sustain circadian rhythms for more than 20 cycles (Yoo *et al.* 2004). The PER2::LUC protein replaced the endogenous protein in mouse tissues and allowed for real-time reporting of circadian dynamics in peripheral tissues.



**Figure 1.3** SCN entrainment of slave oscillators (a) and persistence of rhythmicity of SCN versus liver explants in culture (b). Reprinted by permission from Macmillan

Publishers Ltd: Nature. Reppert, S. M. and Weaver, D. R. Coordination of circadian timing in mammals. *Nature*, 2002, 418: 935-41. © 2002, from Elsevier: Liu, C. and Reppert S.M.: GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron* 2000, 25:123-8 and from AAAS: Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H.: Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 2000, 288:682-5.

The pivotal advance in understanding the dynamics of peripheral clocks was made when bioluminescence imaging of fibroblasts revealed that individual cells could oscillate robustly and independently for an indefinite time but with diverse circadian periods (Welsh *et al.* 2004). As a consequence of this work, the previously observed dampening of peripheral circadian rhythms *ex vivo* is now understood to reflect a gradual desynchrony of many independent cellular oscillators (Welsh *et al.* 2004), thus redefining the role of the SCN as an orchestrator rather than a driver of the circadian symphony. Additional studies using cultured rat fibroblast cell lines revealed that the component oscillators within individual cells could be temporarily resynchronized by a number of different methods, most commonly by a change of culture medium to one containing high serum concentration (Balsalobre *et al.* 1998; Balsalobre *et al.* 2000) (Figure 1.3).

While many substances are known to reset rhythmical expression of clock gene expression in cell culture (Akashi and Nishida 2000; Balsalobre *et al.* 2000; McNamara *et al.* 2001; Yagita and Okamura 2000), the means by which the SCN communicates the time of day information to synchronize peripheral tissues in the intact organism remains to be fully elucidated. Both humoral (Balsalobre *et al.* 2000; McNamara *et al.* 2001) and neural (Bartness *et al.* 2001) communication pathways have been investigated. One remarkable study involving parabiosis in mice, investigated the effect of vascular exchange in the absence of neural communication on peripheral circadian rhythms of SCN-lesioned animals (Guo *et al.* 2005). The results indicated that the SCN regulates expression of circadian oscillations in different peripheral organs by diverse pathways. The finding that neural input is not

required for rhythmic clock gene expression in liver and kidney was not conclusive however, as it is possible that the SCN-intact animal imposed activity and body temperature rhythms on its parabiotically linked partner (Guo *et al.* 2005). Indeed, an indirect method of SCN entrainment of peripheral tissues occurs via direct regulation of the rest/activity cycle, which in turn influences feeding behavior and liver function (Damiola *et al.* 2000;Stokkan *et al.* 2001).

Synchronization of peripheral clocks by the master clock in the SCN ensures that each tissue can adapt its specific function to the correct time of day by means of tissue-specific circadian regulation of transcription (Desai *et al.* 2004;Kita *et al.* 2002;Panda *et al.* 2002;Storch *et al.* 2002;Yamamoto *et al.* 2004;Zambon *et al.* 2003). For example, mRNA's relating to metabolism and detoxification were found to be differentially expressed in microarray analyses of the liver (Akhtar *et al.* 2002;Kita *et al.* 2002). Moreover, very little overlap was found between groups of circadian regulated genes identified in the heart and the liver in one study (Storch *et al.* 2002), and between the liver and the SCN in another (Panda *et al.* 2002). These findings support a specialized role of circadian clocks in each tissue. Under certain conditions, alternative environmental stimuli can override the signal from the SCN. In addition to SCN regulated gene expression under conditions where light is the prevailing entraining agent, non-photoc entrainment by exercise (Buxton *et al.* 2003;Zambon *et al.* 2003) and feeding (Damiola *et al.* 2000;Stokkan *et al.* 2001) have been shown to uncouple peripheral clocks in muscle and liver respectively, and influence gene expression profiles in those tissues. Furthermore, recent findings also suggest that activation of the innate immune system may act as a resynchronizing signal for peripheral clocks (Marpegan *et al.* 2005).

#### **1.4. Circadian-Immune Interactions**

The immune system provides an organism with the means to protect and defend its physiological status quo. The concept of homeostasis is deceiving, as not only does it encompass the mechanisms that react to maintain a constant, fixed set point of a physiological variable (reactive homeostasis), but it is also involves those

mechanisms that are active in advance to maintain a set point that in itself is rhythmic (Moore-Ede 1986). Hence, the ability to adaptively anticipate predictable changes in the environment, as conferred by the circadian system, is an integral component of homeostasis and consequently, interaction between these two systems is fundamental to survival.

Rhythmic secretion of neuroendocrine hormones imposes extensive circadian regulation on immune function (Petrovsky 2001). The circadian rhythm of cortisol, the major circulating human glucocorticoid, is well defined (Leproult *et al.* 2001; Van Cauter and Refetoff 1985; Weibel and Brandenberger 2002; Welsh *et al.* 1995), as is its powerful immuno-suppressant activity (Petrovsky *et al.* 1998; Scheinman *et al.* 1995). Glucocorticoids are potent inhibitors of inflammatory mediators (Russo-Marie 1992) and the ability of cortisol to suppress the pro-inflammatory cytokines IFN- $\gamma$ , IL-12, TNF $\alpha$ , IL-1, and to a lesser extent, IL-6 and IL-10 production, as evident by their reciprocal temporal presence in circulation under normal physiological conditions, has been described (Petrovsky *et al.* 1998). This finding of cytokine production that is negatively entrained by cortisol explains why the symptoms of immuno-inflammatory disorders, such as rheumatoid arthritis and asthma (Bush 1991; Harkness *et al.* 1982; Martin *et al.* 1991; Reinberg *et al.* 1963), are exacerbated at the time of the early morning nadir in plasma concentrations of this hormone (Petrovsky and Harrison 1997).

The immunomodulatory action of the pineal hormone melatonin is also well-established (Colombo *et al.* 1992; Morrey *et al.* 1994) and numerous studies have described the effects of photoperiod on the immune system (Nelson 2004). The presence of melatonin receptors in immune organs and cells further supports a relationship between melatonin and the immune system (Barjavel *et al.* 1998; Rafii-El-Idrissi *et al.* 1996; Rafii-el-Idrissi *et al.* 1995; Yu *et al.* 1991). An important finding is that exogenous melatonin can improve the outcome of acute and chronic inflammation. This immunosuppressant effect is elicited partly via the hormone's ability to inhibit TNF $\alpha$  levels (Wu *et al.* 2001) and reduce levels of IL-6 (Sullivan *et al.* 1996) in mouse models of endotoxin-induced inflammation.

In addition to a hormonal influence on immune system dynamics and circadian regulation of pro-inflammatory cytokines (Petrovsky and Harrison 1997; Petrovsky *et al.* 1998), recent evidence also highlights diurnal changes in circulating levels of different leukocyte populations (Born *et al.* 1997; Kusanagi *et al.* 2004). Furthermore, persistent circadian oscillations of clock genes, cytolytic factors and cytokines were demonstrated in natural killer (NK) cells (Arjona and Sarkar 2006), critical components of the immune system's surveillance against infection and malignancy.

While there is extensive evidence for circadian regulation of immune parameters, there are also strong suggestions that the immune system can modify the central clock. The 'sickness behavior' (Kent *et al.* 1992) displayed by animals in association with antigenic challenge is mediated by the neural effects of cytokines (Dantzer and Kelley 1989). The nonspecific symptoms that accompany the response to infection include anorexia, depressed activity, increased sleep and reduced body care activities (Larson 2002) and are reflections of central circadian disorganization (Cardinali and Esquifino 2003). These responses are thought to be part of a natural homeostatic mechanism the body uses to fight infection (Hart 1988). As previously suggested (Kelley *et al.* 2003), changes in an organism's motivational priorities promote resistance to pathogens by reducing activities that are metabolically expensive (e.g. foraging and grooming) in favor of a state that reduces heat loss (e.g. rest) and increases heat production (e.g. shivering). It is therefore within reason that disruption of circadian behaviors during an immune challenge may serve as an important adaptive response necessary to restore the internal status quo (Johnson 2002). For this reason, cytokine-induced sickness behavior is a good example of a cooperative circadian-immune response.

The first response of the immune system to an antigenic challenge is induction of acute inflammation. The inflammatory response is elicited by neutrophils and macrophages via recognition of pathogen associated molecular patterns (PAMPs) on bacterial and fungal surface elements (Vasselon and Detmers 2002). Toll-like receptors (TLR) recognize these PAMPs and induce intracellular signaling pathways that result in the release of reactive oxygen species, lipid mediators and cytokines

(Diks *et al.* 2001). The endotoxin lipopolysaccharide (LPS) is a component of the outer membrane of gram negative bacteria that has been used extensively in human and animal studies of acute systemic inflammation. LPS binds to LPS binding protein in circulation and this complex then interacts with CD14 on leukocyte cell membranes (Hailman *et al.* 1996; Triantafilou and Triantafilou 2005). CD14 then associates with TLR4 and TLR4 signaling is ultimately responsible for the production and release of inflammatory mediators from activated leukocytes in response to LPS (Diks *et al.* 2001; Sabroe *et al.* 2002). Cytokine to brain communication is thought to be responsible for the sickness behavior observed in response to LPS (Kelley *et al.* 2003; Watkins *et al.* 1995). For example, low dose LPS administration in horses has been shown to induce an acute but transient inflammatory response characterized by tachycardia, lethargy, fever and increased respiratory rate, thought to be associated with TNF $\alpha$  activity (MacKay *et al.* 1991). Differential gene expression profiling in whole blood during LPS-induced acute systemic inflammation in rats highlighted the dramatic temporal changes in gene expression that occurs in leukocytes during an innate immune response (Fannin *et al.* 2005). In addition, significant changes in blood leukocyte kinetics have been shown to occur following LPS administration in humans (Richardson *et al.* 1989).

While it is well established that LPS induces autonomic, endocrine and behavioral responses that are controlled by the brain (Linthorst and Reul 1998; Matsunaga *et al.* 2000), there is limited information regarding the role of the circadian system during acute systemic inflammation. Of particular interest are the reports of circadian clock gene responses to endotoxin challenge in mice. Peripheral LPS administration has been shown to dramatically change immunoreactivity patterns within the SCN in association with changes in locomotor activity rhythms (Marpegan *et al.* 2005). Of greater significance perhaps is the recent finding that the clock gene *Per2* regulates interferon gamma production by NK cells in response to LPS-induced endotoxic shock (Liu *et al.* 2006). However, a role for clock genes in the regulation of other immune cell populations during inflammation has not been investigated.

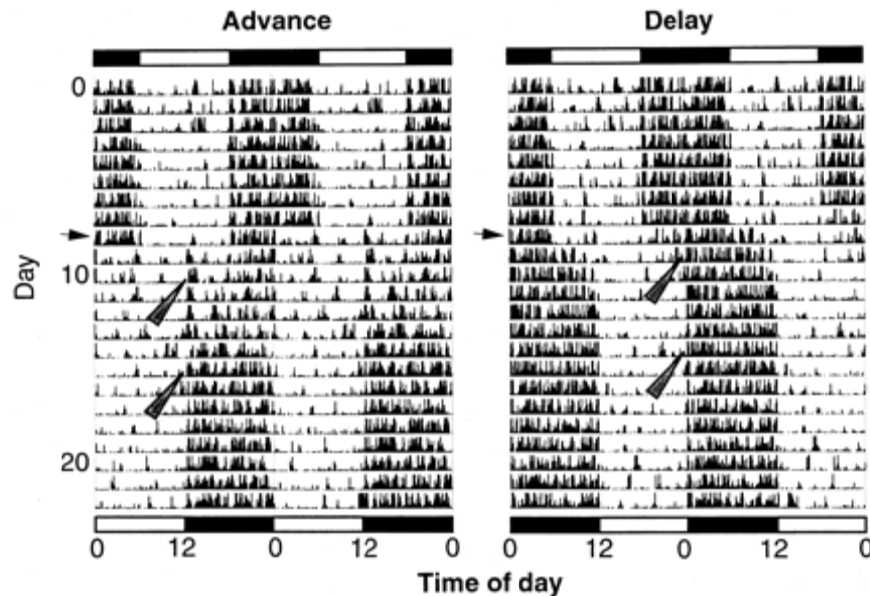
## 1.5. Factors that Disrupt Circadian Timing

Three of the most common factors that disturb circadian timing in humans are transmeridian travel, shift work and genetic mutations in clock or clock related genes. Edison's invention of the incandescent light bulb in 1879 (Israel, 1998) and the introduction of the first commercial jet liner by British Overseas Aircraft Corporation in 1949 (Heppenheimer, 1995) were pivotal societal advances that heralded the beginning of a 24-h society on a much more accessible world. However, artificial time cues associated with revolving shift work and rapid transmeridian travel pose similar challenges to a circadian system evolved to deal with gradual seasonal changes in daylength provided by the natural environmental light/dark cycles. The temporal disorder of physiology experienced during these conditions is a result of a conflict between the new cycle of light and dark and the previously entrained program of the internal clock. Jet lag is the term used to describe the combined symptoms of malaise, appetite loss, fatigue and disturbed sleep associated with rapid displacement across the earth's time zones (Loat and Rhodes 1989). These symptoms last until the circadian clock system adjusts to the new environmental conditions, re-establishing preferred phase relations among different rhythms and between these rhythms and the external environment (Gander *et al.* 1985; Sato *et al.* 2004). The rate of adjustment to a new time zone depends on the circadian output rhythm being measured, the number of time zones crossed, the flight direction (eastward or westward), and the strength of the entrainment factors (i.e. light cues) in the new time zone (Gander *et al.* 1985; Loat and Rhodes 1989).

Understanding circadian readjustment to acute time zone transitions requires an analysis of the molecular and cellular events that occur both in the SCN and peripheral tissues during resetting. Prolonged circadian disturbances are associated with eastward travel, or phase advances, as first described by Aschoff (Aschoff *et al.* 1975) and later demonstrated at the molecular level (Reddy *et al.* 2002). Advances in the photoschedule are characterized by gradual resetting, during which the activity-rest cycle of rats and mice take several days to achieve a steady-state shift, whereas



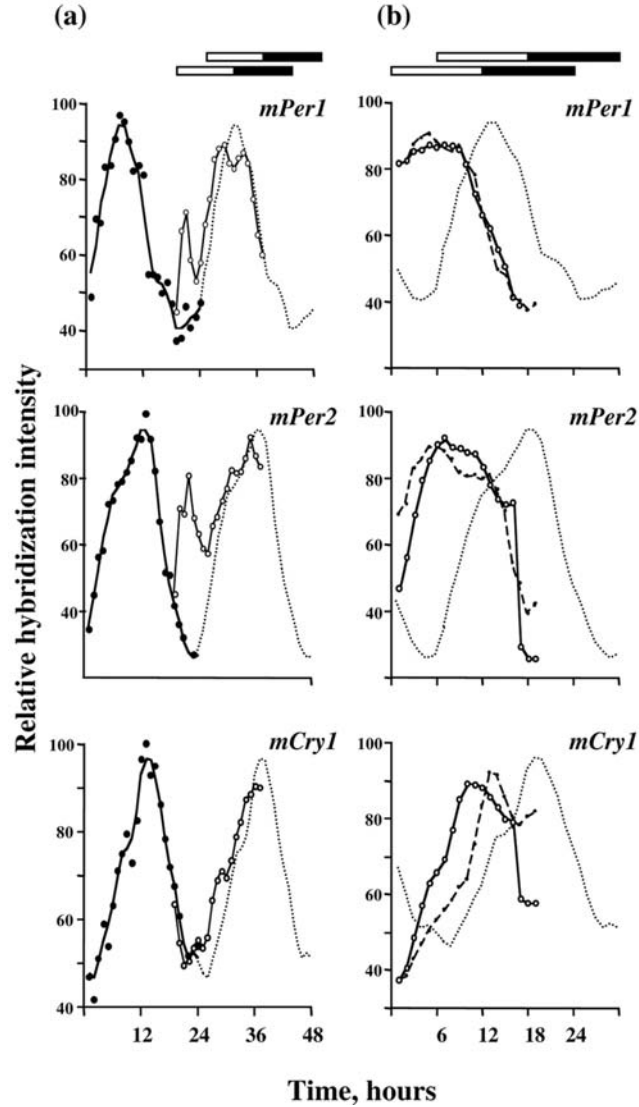
delays are executed rapidly (Reddy *et al.* 2002; Yamazaki *et al.* 2000) (Figure 1.4). The reason that phase advances are less well tolerated by the circadian system is likely due in part to two key factors. Firstly, the intrinsic free-running period of the human circadian system is slightly longer than 24 h, making delays more amenable than advances (Aschoff *et al.* 1975). Secondly, the *Period* components of the molecular clock in the SCN are rapidly induced by light during early night (Miyake *et al.* 2000; Shearman *et al.* 1997; Yan *et al.* 1999), potentially explaining how the circadian system resets more readily when exposed to the lengthened hours of afternoon light associated with westward transitions.



**Figure 1.4** Representative locomotor activity records from rats in response to a 6-h phase-advanced or phase-delayed light cycle. General activity was monitored with an implanted transmitter and has been double plotted. The original LD cycle (black and white bars at top of figure) was shifted either 6 h earlier (left panel) or 6 h later (right panel) on the day indicated by the arrow (new light cycle indicated by black and white bars at bottom of figure). Complete re-entrainment as measured by visual inspection took about  $6.2 \pm 0.5$  cycles (SEM,  $n = 10$ ) following the phase advance or  $1.8 \pm 0.3$  cycles ( $n = 10$ ) following the phase delay. From Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M.

and Tei, H. Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, 2000, 288: 682-5. Reprinted with permission from AAAS.

As previously described (section 1.2), the core molecular clock components *Bmal1* and *Per2* have circadian oscillations that are antiphase to each other (Yagita *et al.* 2001). *Cry1*, under normal circumstances, oscillates in phase with *Per2* i.e. both transcripts reach peak amplitude at the same time in the circadian cycle. However, during light phase advances or delays, such as can be experienced during eastward or westward travel, a dissociation of the cycles of expression of *Cry1* from *Per2* within the SCN has been reported (Reddy *et al.* 2002) (Figure 1.5). Reddy *et al.* (2002) demonstrated that circadian cycles of *mPer2* expression in the mouse SCN react rapidly to an advance in the lighting schedule, whereas rhythmic *mCry1* expression requires 8 days to complete the advance. It was suggested that this dissociation is the origin of inertia during resetting and results in the temporal disorder of physiology experienced as jet lag (Reddy *et al.* 2002).



**Figure 1.5** Re-entrainment of SCN gene expression cycles to an advanced lighting schedule. **a**, Intensity of mRNA hybridization signals for *mPer1*, *mPer2*, or *mCry1* in SCN before (baseline) and on the first day of the advance phase shift. Baseline data are plotted as observed hourly values (*closed circles*, mean of 3 mice/h), and as the three-point moving average (*solid line*, double-plotted on *right* as *dotted line* for clarity). Data from day 1 of the shift are single-plotted on the right (*open circles*, *solid line*), represented by the observed hourly mean (3 mice/h, for the first 6 h of light exposure) or as the three point moving average for the hourly means (3 mice/h) for the subsequent 12 h. **b**, Intensity of SCN mRNA hybridization signals before (baseline data, *dotted line*) and on the third (*closed circles*, *dashed line*) and eighth

(*open circles, solid line*) days of the advance phase shift. From Reddy *et al.* 2002. Reprinted with permission from the Society for Neuroscience.

More recently, two spatially distinct oscillators have been discovered within the SCN (Nagano *et al.* 2003), providing an alternative explanation for the physical malaise associated with jet lag. Nagano *et al.* (2003) demonstrated dissociation of synchronous clock gene oscillations within subregions of the SCN in response to abrupt phase advances or delays of the LD cycle. Clock gene expression rhythms within the ventrolateral, photoreceptive region of the SCN entrained rapidly to the new LD cycle, while those of the dorsomedial, non-photoreactive region shifted slowly. This gradual re-entrainment within the dorsomedial region was correlated with, and mirrored, the disruption in circadian behavioral output, as measured by locomotor activity rhythms. These results led the authors to suggest that this dissociation between SCN subregions may be responsible for the desynchrony between overt behavior and environmental LD cycles under jet lag conditions.

In addition to desynchronized clock gene expression within the master pacemaker in the SCN, it is logical that rhythmic gene expression in peripheral tissues, which rely on SCN signals for synchrony, would also be significantly disrupted. This was in fact clearly demonstrated in rats (Yamazaki *et al.* 2000). Yamazaki *et al.* (2000) reported that rhythmicity in skeletal muscle, liver and lung shifted more slowly than the SCN following both light cycle advances and delays and concluded that this is likely to further explain the physical malaise associated with rapid transmeridian travel.

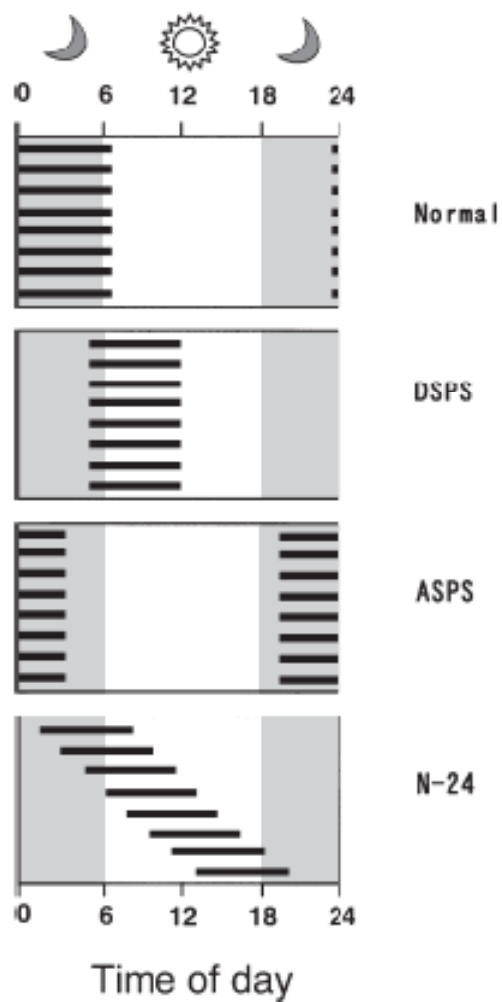
Jet lag is of particular concern to athletes hoping to perform optimally at an international destination. A comparative analysis of bi-directional effects of time zone displacement on human athletic performance was first conducted in 1966 (Hauty 1967; Hauty and Adams 1966). Results of this early study clearly demonstrated disturbances in heart rate, respiratory rate, body temperature, evaporative water loss and psychological function, and again the disturbances were found to be more profound following an easterly flight (phase advance) (Hauty 1967). Later studies demonstrating prolonged physiological disturbances following eastward transition

supported these early findings (Klein and Wegmann 1974; Klein *et al.* 1972; Wever 1980). Further validation was provided by a much more recent study in athletes from the German Olympic team (Lemmer *et al.* 2002). The primary aim of the study was to investigate the effects of eastward and westward transmeridian flight on heart rate (HR) and blood pressure (BP) profiles from the point of view that, in athletes, BP and oxygen supply to the organs are of utmost importance for optimal performance and successful competition. In addition, rhythms in body temperature, saliva melatonin, cortisol and grip strength were determined. After both flights the rhythmic patterns in body temperature and grip strength were greatly disturbed. Resynchronization of all rhythms was not complete until day 11 after time zone transition (Lemmer *et al.* 2002). Another study using fit human subjects examined performance times before and after an eastward journey across 6 time zones (Wright *et al.* 1983). Performance times for a 270 m sprint were found to be slower for the first 4 days following translocation as were times for a 2.8km run on the second and third days. This reduction in performance capacity was suggested to be due to several neuromuscular, cardiovascular and metabolic variables and indices of aerobic capacity that are out of synchrony with the environmental light-dark cycle following a transmeridian journey (Winget *et al.* 1985). The recent evidence indicating loss of clock gene rhythmicity in lung, muscle and liver tissue following abrupt LD phase shifts (Yamazaki *et al.* 2000) provides a molecular explanation for such performance deficits. However, it is also likely that stress associated with travel compounds these effects.

Several well-characterized circadian rhythm sleep disorders occur as a result of polymorphisms in clock genes that cause diurnal changes in human behaviors (Ebisawa 2007) (Figure 1.6). These include a single nucleotide polymorphism located in the 3' flanking region of the *Clock* gene that is reportedly associated with human diurnal preference towards morningness or eveningness (Katzenberg *et al.* 1998). Familial advanced sleep phase syndrome (ASPS), a disorder where affected individuals are "morning larks" with a 4-h advance of the sleep, temperature, and melatonin rhythms, has been shown to be associated with two separate mutations. One occurs in the *casein kinase I (CKI) epsilon* binding region of *Per2* and results in hypophosphorylation in vitro (Toh *et al.* 2001). A second missense mutation found to

result in this disorder occurs in the *CKI delta* gene and reduces phosphorylation activity of the enzyme (Xu *et al.* 2005). Furthermore, a missense polymorphism in the *Per3* gene has been shown to be a risk factor for delayed sleep phase syndrome (DSPS) (Ebisawa *et al.* 2001). Similarly, a single nucleotide polymorphism in the rate limiting enzyme in melatonin synthesis has also been implicated in this sleep disorder (Hohjoh *et al.* 2003). The final schematic in Figure 1.6 shows the free-running sleep pattern associated with non-24-h sleep-wake disorder. This sleep disorder is typical of blind people with both eyes enucleated such that ocular light transmission from the retina to the SCN is blocked (Skene and Arendt 2007).

An additional sleep disorder not associated with genetic abnormalities is shift work sleep disorder (SWSD), classified as a disorder of excessive sleepiness or insomnia (American Academy of Sleep Medicine, 2005). The importance and far-reaching implications of circadian misalignment in shift workers is best illustrated by an example of the disasters resulting from severe fatigue at night: the Exxon Valdez oil spill in Alaska's Prince William Sound; the nuclear reactor meltdown in Chernobyl, Russia; the chlorine gas release in Bhopal, India; the radiation gas cloud at Three Mile Island, Pennsylvania; and NASA's Challenger disaster (Medscape Today, 2005).



**Figure 1.6** Schematic diagrams showing sleep patterns in human circadian rhythm sleep disorders. Thick lines indicate sleeping hours. DSPS – Delayed Sleep Phase Syndrome; ASPS – Advanced Sleep Phase Syndrome; N-24 – Non 24 h Sleep Phase Syndrome. From Ebisawa, T. Circadian rhythms in the CNS and peripheral clock disorders: human sleep disorders and clock genes. *J Pharmacol Sci*, 2007, 103: 150-4. Reprinted with permission from the the Japanese Pharmacological Society.

### 1.6 Markers of Circadian Phase

The precise cellular, organismal, and behavioral mechanisms underlying jet-lag and shift work induced reductions in physiological performance remain to be elucidated. Steps towards understanding these phenomena have involved

characterizing the severity and longevity of measurable perturbations in normal circadian organization, as can be observed by studying and quantifying the parameters of two or more well characterized circadian rhythms in relation to each other and to the LD cycles employed. Plasma levels of melatonin and cortisol, and core body temperature readings have historically been used as markers of circadian phase position (Benloucif *et al.* 2005;Klerman *et al.* 2002;Lewy and Sack 1989). In mammals, there is evidence of an inverse relationship between plasma melatonin and cortisol circadian rhythms (Weibel and Brandenberger 2002). Melatonin secretion from the pineal gland is highest during the hours of darkness, declines in the early morning and stays low during the daytime. In contrast, the 24-h pattern of plasma cortisol concentration peaks in the early morning, declines in the afternoon and remains low most of the night. Weibel and Brandenberger (2002) found that there was a significant correlation between the timing of the melatonin onset and the timing of the start of the quiescent period of cortisol secretion. The authors demonstrated that this relationship remains phase-locked in subjects working night shifts despite phase shifts of the hormonal rhythms. It was concluded that the quiescent period of cortisol and melatonin onset were two coordinate markers and suggested their use as reliable determinants of circadian phase. However, while robust rhythmical secretion of melatonin has been documented in the horse (Piccione *et al.* 2005), analysis of 24-h cortisol secretion revealed less stable rhythms that were highly susceptible to slight environmental changes in this species (Irvine and Alexander 1994).

Body temperature is another commonly assessed marker of circadian phase (Benloucif *et al.* 2005;Klerman *et al.* 2002;Refinetti and Menaker 1992) and has been used to determine rates of re-entrainment to LD phase shifts in humans (Boivin and James 2002) and rodents (Goel and Lee 1996). The robust nature of the core body temperature rhythm of the horse was demonstrated by Piccione *et.al.* (2002) under a light-dark cycle and in constant illumination. The persistence of the body temperature rhythm under constant light confirmed the endogenous nature of the rhythm in this species (Piccione *et al.* 2002).



## **1.7. Research Objectives**

**Chapter 2:** To identify, sequence and FISH map the equine core circadian clock genes.

**Chapter 3:** To design quantitative Real Time RT-PCR assays necessary to investigate the temporal relationships of clock gene expression in an equine fibroblast cell line, peripheral blood and adipose tissue.

**Chapter 4:** To determine if induction of acute systemic inflammation transiently synchronizes clock gene expression in equine peripheral blood and identify potential immune mediators involved.

**Chapter 5:** To identify the cell type responsible for clock gene upregulation during acute systemic inflammation and investigate associated blood cell population kinetics.

**Chapter 6:** To investigate the re-entrainment rates of two markers of equine circadian phase, serum melatonin and core body temperature, following an abrupt 6-h advance of the light/dark schedule, mimicking a transmeridian journey across 6 time zones.

**Chapter 7:** Conclusions and future directions.

## CHAPTER 2

### Isolation, Sequencing and *Fluorescence In Situ Hybridization* (FISH) Mapping of the Core Circadian Clock Genes in the Horse

#### 2.1 Summary

The mammalian molecular clock is responsible for circadian rhythms of physiology and behavior. It is comprised of multiple gene-protein-gene feedback loops whereby proteins have a negative effect on their own transcription while simultaneously stimulating the transcription of other clock genes. Four core circadian clock genes involved in the feedback loops responsible for the perpetuation of cellular rhythmicity are *Bmal1*, *Cry1*, *Per2* and *Clock*. This chapter reports the successful isolation of cDNA fragments corresponding to the equine homologs of these genes, including the complete coding sequence for equine *Bmal1*. The National Center for Biotechnology Information (NCBI) Blast analysis of sequencing results revealed remarkably close homology between the equine clock genes and their human counterparts. Of particular interest is equine *Bmal1*, whose deduced amino acid sequence demonstrated 99% homology with its human counterpart. Examination of the nucleotide sequence of *Bmal1*, however, revealed a novel 140 base pair region within the 5'-UTR that appears to be unique to the horse. In addition, the chromosomal locations for these equine core clock genes were determined by *fluorescence in situ hybridization* (FISH). *Bmal1* is located on ECA7q16-q18, *Per2* on ECA6p12-p13, *Clock* on ECA3q21 and *Cry1* on ECA28q15-q16. The addition of four Type 1 markers to the equine physical map has contributed to comparative mapping between horse and human and will aid inheritance studies relating to the equine circadian system.

[Some of the material in this chapter has been published in *Animal Genetics* 2007, 38:84-85.]

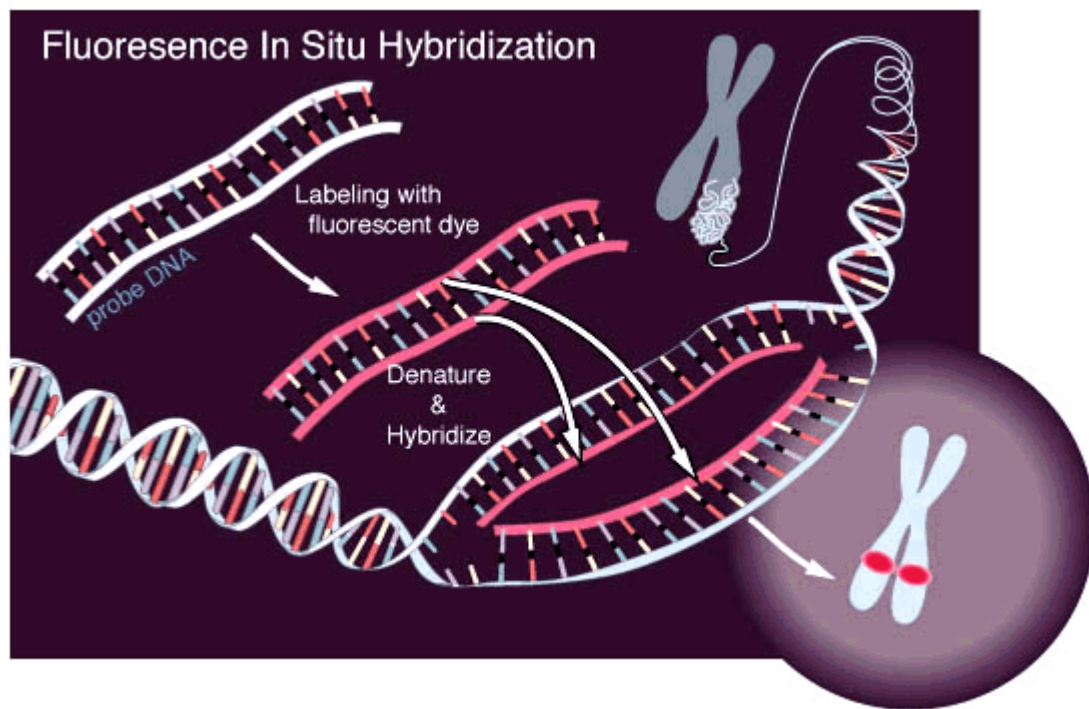
## 2.2 Introduction

Almost all organisms on earth are exposed to the daily light/dark cycles imposed by our revolving planet and thus circadian rhythms represent an integral feature of life. Cellular clock mechanisms have remained remarkably conserved across species (Hsu *et al.* 1996; Shigeyoshi *et al.* 2002; Takahashi 2004) throughout evolution, from the lowest invertebrates to mammals, due to the persistent, 24-h periodic nature of the earth's revolutions and the evolutionary pressure to optimize survival by adapting physiological function relative to the photoperiod. The core oscillator components of the molecular clock have been well characterized in humans and rodents, but to a much lesser degree in large domesticated species (Takata *et al.* 2002; Yamamoto *et al.* 2004). To date, clock gene sequence information along with functional expression data has only been reported for the sheep (Andersson *et al.* 2005). Isolation and sequencing of the equine core clock genes is a necessary first step for investigating clock gene expression, enabling our understanding of circadian regulation at the molecular level in the horse. The primary goal of this study was to generate quality cDNA sequence suitable for the design of quantitative (q) reverse transcription (RT) polymerase chain reaction (PCR) assays to detect transcript levels of the equine clock genes. An additional goal was to determine how closely related clock genes are between horse and human. Within the mammalian molecular clock, CLOCK and BMAL1 heterodimerize to provide the positive drive for the transcription of *Period* (*Per 1-3*) and *Cryptochrome* (*Cry 1-2*) genes, and these in turn form complexes that negatively regulate their own transcription (Reppert and Weaver 2001). Equine homologs of *Clock*, *Bmal1*, *Per2* and *Cry1* were chosen for isolation and sequencing as they are core components of the mammalian circadian clock, representing both the negative and positive feedback loops of the clockwork mechanism.

At the time this study was initiated, available equine specific sequence data was limited. The standard procedure for the isolation of new equine transcripts was to design oligonucleotide primers (nucleic acid strands that serve as starting points for DNA replication) from conserved sequence regions in multispecies cDNA alignments

of the gene of interest. The premise being that regions of a specific gene transcript that have remained conserved between mouse and human, for example, would likely also be conserved in the horse, and would permit successful amplification of the product when probing an equine specific cDNA library by PCR. The resulting PCR products would subsequently be sequenced and the nucleotide sequence compared against the existing NCBI nucleotide sequence database to confirm the identity of the product as the equine homolog of the gene of interest.

A final goal of the work presented in this chapter was to assign the equine clock genes to specific equine chromosomes by FISH, a cytogenetic technique used to detect and localize the presence or absence of particular DNA sequences on chromosomes (Figure 2.1).



**Figure 2.1** Overview of FISH technique. Illustration courtesy of National Human Genome Research Institute (NHGRI) (<http://www.genome.gov/>).

## 2.3 Methods

### 2.3.1 *Clock gene Isolation and Identification*

Oligonucleotide primers for polymerase chain reaction (PCR) were designed from conserved regions in alignments between mouse and human cDNA sequences for *Bmal1*, *Per2*, *Clock* and *Cry1*. The primers were used to amplify the equine homologs of the clock genes by PCR from both a prepared equine lymphocyte cDNA library and an equine brain cDNA library (courtesy of Dr. Lois A. Wetmore). The PCR was performed using FastStart DNA polymerase (Roche, Indianapolis IN) in a 20µL volume containing 80ng cDNA template, .5 µM forward and reverse primers and all other reagents according to the manufacturer's recommendations. Primer sequences, annealing temperatures and product sizes are shown in Table 2.1. Products were visualized by electrophoresis on a 1% agarose gel after staining with ethidium bromide. Product bands of the correct size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Prior to sequencing, gel purified products were amplified using BigDye Terminator v1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA), cleaned using Centri-Sep columns (Princeton Separations Inc., Adelphia, NJ), and run on an ABI 310 genetic analyzer (Applied Biosystems) according to the manufacturer's recommendations. For products longer than 500 base pairs, internal primers were designed for sequence walking in both the forward and reverse direction (Table 2.2), ensuring at least a two-fold coverage of the sequence. All chromatograms were visually inspected for base calling errors. Fragments of the same gene were assembled using the ContigExpress program of the Vector NTI Suite 9 software package (InforMax Inc., Frederick, MD).

**Table 2.1** Primers used for PCR to identify equine clock genes

Gene	Sequence 5'-3'	Product length in bp	Annealing Temp. °C
<i>Bmal1</i>	F: AGGGATAAAAAATGCAAGGGAAGCTCA	1986	60
	R: CCAATGATGCTTCTGTGCACAATGATTAAA		
<i>Per2</i>	F: GCAGTAGTGACACAAGTCATAC	187	55
	R: GCAGCTGGTACGTCATCATGACGCTG		
<i>Cry1</i>	F: GCATCAACAGGTGGCGATTTTTGC	1174	58
	R: GCAGGGAAGCCTCTTAGGACAGG		
<i>Clock</i>	F: TTTGATGGGTTGGTGAAGAAGATGA	547	56
	R: TGGGTCTATTGTTCTCGCAGCAT		

**Table 2.2** Additional primers used for sequence walking

Name	Sequence 5'-3'	Product length in bp	Annealing Temp. °C
F2Bmal1.cDNA	TCAAGATCCTCAACTACAGCCAGAATG	1324	60
R2Bmal1.cDNA	GCCACCCAGCCCAGCATCT		
F3Bmal1.cDNA	GGGGCTGGATGAAGACAACGAACC	571	60
R3Bmal1.cDNA	CCCCCTGGAATCCCTGGAACA		
EqBmal1F	CAGGGCAGCAGATGGATTTTTGTTTG	1420	57
EqBmal1R	CGGCCATGGCAAGTCGCTAAAGTC		
5'UTRBmal1F	ATGTGGAATCCTGGGCCTTCATT	423	56
EqRev5'Bmal1	TCGTCTTTGTCTGTGTCCATGCTTTC		
Cry1F2	GCCTTCCGCCCATTTCGCT	585	60
Cry1R2	CATAGAACTCAATGGTGGACAGCCA		
Cry1F3	CCGGTTTGGGTGTCTGTCTGTGTC	no product	
Cry1R3	CGGCCACACTGCAGAGGGTAAG		

### 2.3.2 Fluorescence In Situ Hybridization (FISH)

Primers were designed and tested for successful amplification of equine genomic DNA corresponding to *Bmal1*, *Per2*, *Cry1* and *Clock* prior to PCR screening of the CHORI-241 equine bacterial artificial chromosome library (BAC) library (Table 2.3) (screening courtesy of Dr. D.L. Adelson, TAMU). One positive clone for each gene was grown under standard culture conditions and DNA extracted using the Perfectprep kit (Eppendorf, Westbury, NY). The Nick Translation Kit, including

SpectrumGreen-dUTP, SpectrumRed-dUTP and SpectrumOrange-dUTP from Vysis Inc. (Downers Grove, IL) was used to label the extracted BAC DNA according to the manufacturer's recommendations. Probes were hybridized to slides containing equine metaphase chromosomes as previously described (Bellone *et al.* 2006). The slides were provided courtesy of Dr. Teri. L. Lear and were prepared as previously described (Bailey *et al.* 1997). Predictions of equine chromosomal locations were made based on the human megabase positions of each clock gene (Kent *et al.* 2005) and the comparative horse-human map (Perrocheau *et al.* 2006). Based on these predictions, previously published markers (Caetano *et al.* 1999; Chowdhary *et al.* 2003; Mariat *et al.* 2001; Terry *et al.* 2002) were chosen to co-localize with each of the clock genes, which served to confirm their positions on specific equine chromosomes.

**Table 2.3** Primers used to probe the BAC library

Gene	Sequence 5'-3'	Product length in bp
<i>Bmal1</i>	F: GGCCCTGTTGACTTTAGTGACTTGC	270
	R: ATCCACAGCTAGCCCAATAATCAATGA	
<i>Per2</i>	F: CCAGCAAATATTTTCGGAAGCATCGA	127
	R: GCCATCAGCAGCCAGACAGG	
<i>Cry1</i>	F: TGGGCAACTGTTATGGCGTGAATTTT	242
	R: CCCGTGTCAGGAAGCAAGCAACT	
<i>Clock</i>	F: AGTATCTAGAAACAAATCTGAAAAGAAACGTAGA	124
	R: GCTCTTCTGTAGAACAGTAGATTTGTCCATC	

## 2.4 Results

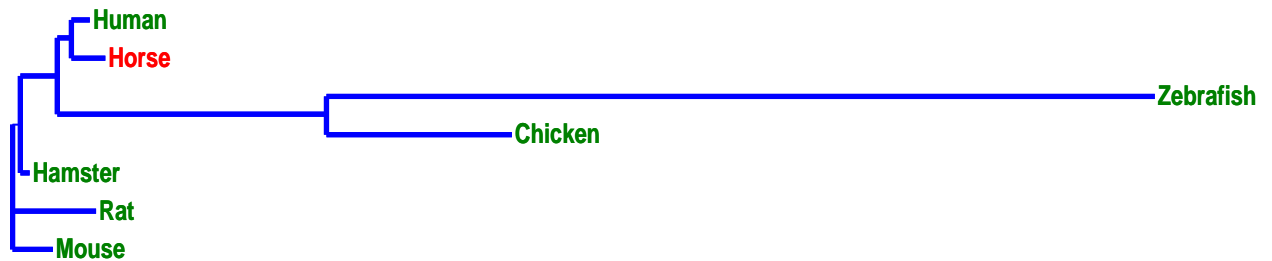
### 2.4.1 Sequencing Results

NCBI Blast analysis of the sequencing results confirmed the identity of each of the PCR products as an equine homolog of one of the clock genes. The partial coding sequence (CDS) of equine *Per2*, *Cry1* and *Clock* and the complete CDS of *Bmal1* have been submitted to Genbank (the NIH genetic sequence database) along with the deduced amino acid sequences. Genbank accession numbers are listed in

Table 2.4 along with the sequence length for each gene and the percent homologies between horse and human. A phylogeny tree constructed using VectorNTI shows the equine BMAL1 protein clustering more closely with the human homolog than that of the hamster, rat or mouse (Figure 2.2)

**Table 2.4** Sequencing results for the equine clock genes and homology to human orthologs as determined by NCBI Blast analysis.

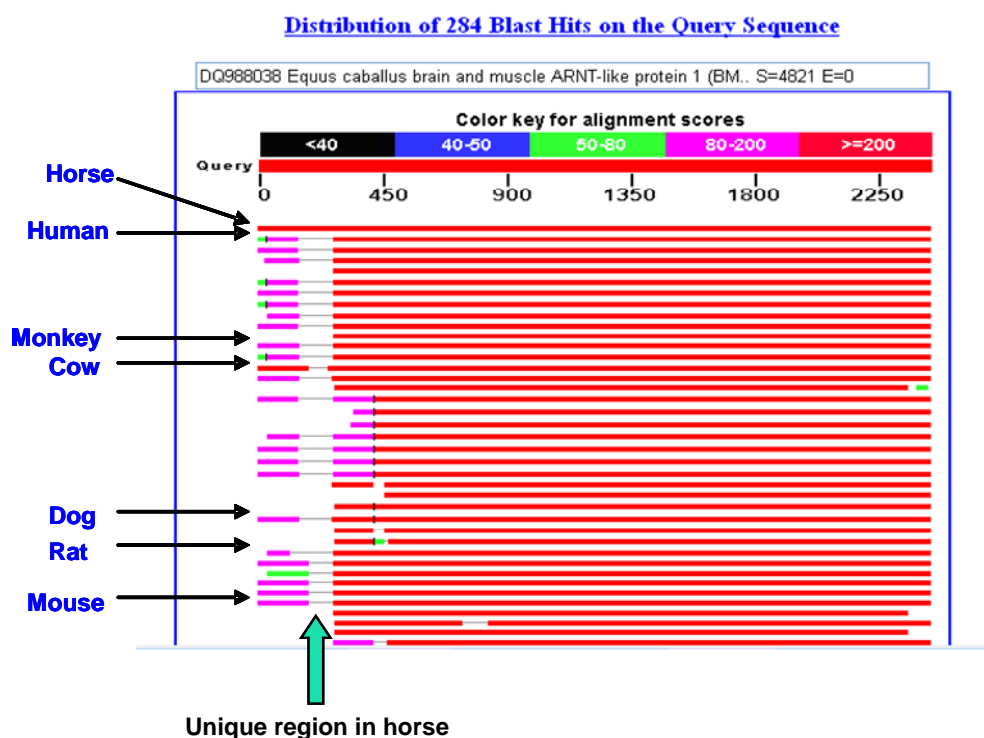
Gene	GenBank Accession No.	Nucleotide Length (bp)	% Nucleotide homology	% Amino acid homology
<i>Bmal1</i>	DQ988038	2432	95	99
<i>Cry1</i>	DQ988039	1135	94	98
<i>Clock</i>	DQ988040	530	98	100
<i>Per2</i>	EF015879	187	82	80



**Figure 2.2** Interspecies phylogeny tree of BMAL1 amino acid sequences created by the Align X program of VectorNTI. This tree is built using the Neighbor Joining method and works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences and do not accurately reflect evolutionary divergence.



Additional sequencing of PCR products from primers designed to amplify the 5'- untranslated region (UTR) of equine *Bmal1* (Table 2.2) permitted completion of the entire CDS for this gene. NCBI Blast analysis of the sequencing results for *Bmal1* revealed that 140 base pairs within the 5'-UTR are unique to the horse when compared with orthologous sequences for multiple other species (Figure 2.3). Within this unique region is the presence of an upstream ATG initiation codon followed by an in-frame stop codon, creating a short upstream open reading frame (ORF) (Figure 2.4). Further analysis of this sequence by submitting it to the UTRsite database did not reveal any known regulatory motifs (Mignone *et al.* 2005).



**Figure 2.3** NCBI Blast results demonstrating an equine specific 5'-UTR insertion



**Figure 2.4** Nucleotide sequence of equine *Bmal1* 5'-UTR. The region unique to the horse is highlighted in blue. One letter amino acid symbols corresponding to the residues within the ORF appear above the relevant sequence. The upstream ATG start site is highlighted in bold and the actual start codon appears in red with a red bar over the putative Kozak consensus sequence.

#### 2.4.2 Chromosomal Assignments for Equine *Bmal1*, *Per2*, *Cry1* and *Clock*

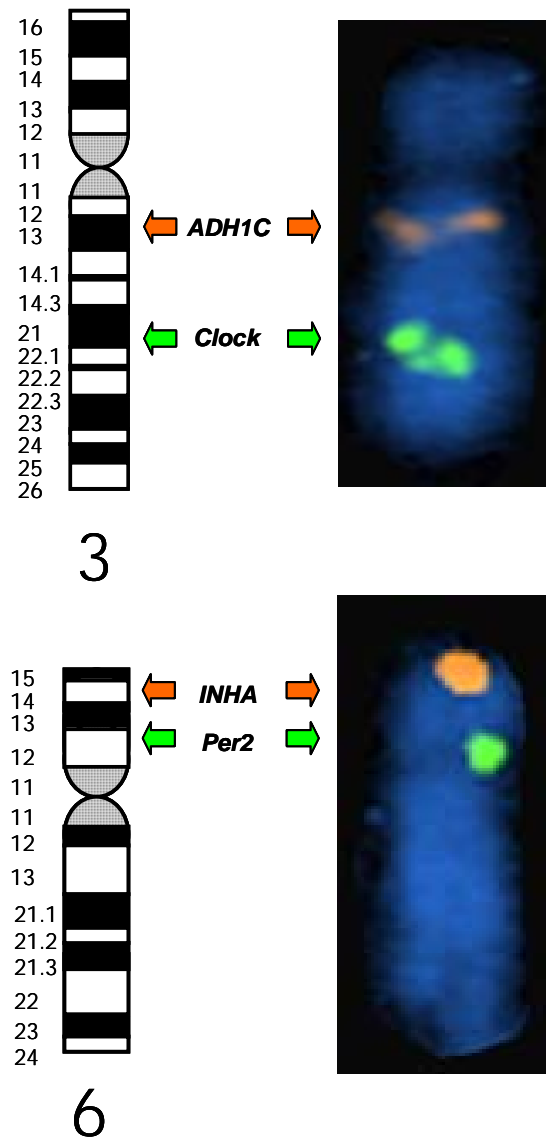
Twenty-five metaphase chromosomes were examined for each gene and it was determined that *Bmal1* is located on ECA7q16-q18, *Per2* on ECA6p12-p13 and *Clock* on ECA3q21 (Figure 2.5). Based on the recently published horse-human comparative map (Perrocheau *et al.* 2006) and the human position of *Cry1* on HSA12:105.9Mb, *Cry1* could have been located on ECA8 or ECA28. Our FISH results determined that *Cry1* maps to ECA28q15-q16 and not to ECA8 (Figure 2.6). FISH results are summarized in Table 2.5.

**Table 2.5** Chromosomal assignment of equine clock genes by FISH

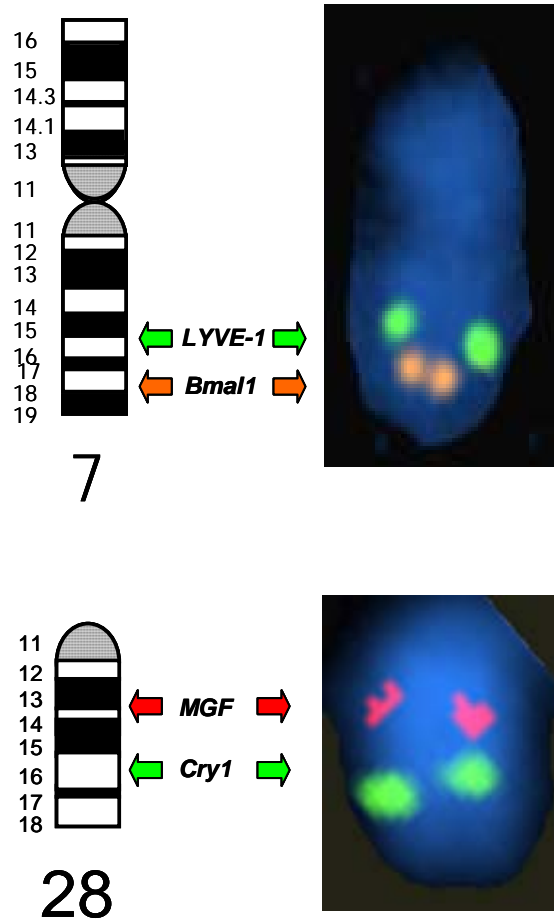
<b>Gene</b>	<b>CHORI-241 clone</b>	<b>Co-localized marker</b>	<b>ECA<sup>1</sup></b>	<b>Conserved synteny to HSA<sup>2</sup></b>
<i>Bmal1</i>	91:D20	LYVE1	7q16-q18	11p15
<i>Per2</i>	44:A20	INHA	6p12-p13	2q37
<i>Cry1</i>	73:I3	MGF	3q21	12q23
<i>Clock</i>	11:A9	ADH1C	28q15-q16	4q12

<sup>1</sup> Equus caballus autosome

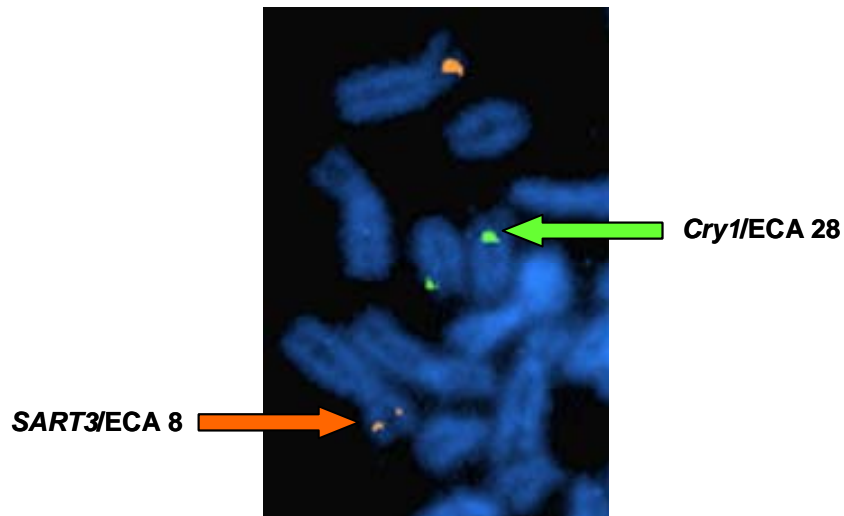
<sup>2</sup> Homo sapien autosome



**Figure 2.5** Ideograms of equine chromosomes and FISH images showing location of clock genes and previously published markers. Ideograms adapted from ISCNH (1997).



**Figure 2.5 continued.** Ideograms of equine chromosomes and FISH images showing location of clock genes and previously published markers. Ideograms adapted from ISCNH (1997).



**Figure 2.6** FISH image of *Cry1* location on ECA 28 and not on ECA 8 as determined by location of ECA 8 marker *SART3* on a different chromosome pair.

## 2.5 Discussion

The primary goal of this work was to isolate the equine clock genes and generate sequence data suitable for design of primers and probes for gene specific quantitative expression assays. To this end, equine *Bmal1*, *Cry1*, *Clock* and *Per2* were successfully isolated and cDNA sequences generated. Analysis of the equine sequences revealed a high level of homology with their human counterparts at the nucleotide and amino acid level. This is further evidence of the high degree of conservation that exists within the core components of the circadian clock across diverse taxonomic classes. The horse is the first member of the order *Perissodactyla* for which circadian clock genes have been reported. The high sequence homology between horse and human also provides impetus to consider the horse as a potential model for investigation of human circadian disorders. Small mammals have historically been used as molecular models to investigate circadian function and disorders in humans. However, behavioral rhythms of diurnal mammals, including humans, are almost opposite those of nocturnal rodents. Investigating circadian regulation in a large diurnal mammal with good sequence homology to the human

clock genes, such as the horse, may provide important comparative information for human studies.

While several attempts were made to amplify larger segments of equine *Per2* cDNA, these yielded poor quality sequence data and in many cases non-specific PCR products, suggesting a lower degree of sequence homology for this gene. Indeed, the nucleotide and amino acid homology between horse and human for *Per2* is significantly lower than for the other three genes. However, an NCBI Blast analysis of human *Per2* revealed that homology between mouse and human is only 80 % at the nucleotide level and 77 % at the protein level (data not shown), still considerably lower than between human and horse.

Isolation of the complete coding sequence for equine *Bmal1* as well as the 3'-UTR plus a significant portion of the 5'-UTR provided another opportunity to compare homology to other species. The discovery of a 140 base pair region within the 5'-UTR of *Bmal1* that is unique to the horse raises important questions about the regulation of this clock transcript in this species. 5'-UTRs are thought to play important roles in the post-transcriptional regulation of gene expression including translation efficiency (van der Velden and Thomas 1999), mRNA stability (Bashirullah *et al.* 2001) and subcellular localization (Jansen 2001), all of which might have ramifications for the gene-protein-gene auto-regulatory feedback loops necessary for the molecular clockwork mechanism, of which *Bmal1* plays a crucial role. While computational scanning of this unique sequence did not reveal any known functional motifs specific to 5'-UTRs, the presence of an upstream ORF may have implications for translational control.

The sequences flanking the ATG initiation start codon form a consensus sequence in mammals known as the Kozak sequence, which facilitates the initial binding of the mRNA to the ribosome. The Kozak consensus sequence is GCCRCCaugG, of which the purine (R) in position -3 and G at position +4 are the most conserved nucleotides (Kozak 1987). As the sequence context of the upstream ATG in the 5'-UTR of equine *Bmal1* makes it a poor initiation codon, it is likely that it is often bypassed by the ribosomal subunit in favor of the more distal ATG. However, "leaky scanning" may occur where multiple proteins are obtained from the

same mRNA (Xiong *et al.* 2001) possibly reducing the basal translational level of the gene. The process of translating an upstream ORF is also thought to down-regulate translation of the mRNA (Vilela *et al.* 1999) as it impairs translation of the main ORF by hampering the ability of the ribosome to reinitiate at the next start site (Luukkonen *et al.* 1995). The potential functional significance of this unique insertion in equine *Bmal1* may be worth re-evaluating later, should expression analysis of the clock genes demonstrate altered temporal profiles in this species.

Interestingly, the predicted amino acid sequence of bovine BMAL1 shows greatest homology to the equine 5'-UTR, closing the gap on the segment unique to the horse by approximately 60 nucleotides. This suggests that this region may have particular importance for regulation of *Bmal1* transcription in ungulates.

Finally, the four equine clock genes were successfully FISH mapped to equine chromosomes, contributing to comparative mapping between horse and human. In particular, it was determined that *Cry1* maps to ECA 28q15-q16 and not to ECA 8, thereby extending the conserved synteny of this portion of HSA12q to include an additional 4.5Mb on ECA 28. Increasing the density of the equine FISH map by addition of four Type 1 markers provides a valuable reference for construction of the genome assembly, which is currently underway, as FISH markers provide concrete evidence for the physical position of genes on the genome.



## CHAPTER 3

### Investigation of Clock Gene Expression in Equine Peripheral Tissues

#### 3.1 Summary

The master mammalian pacemaker in the brain controls numerous diverse physiological and behavioral processes throughout the organism. Timing information is continually transmitted from the master clock to peripheral organs to synchronize rhythmic daily oscillations of clock gene transcripts and control local physiological function. To investigate the presence of peripheral clocks in the horse, quantitative Real-Time RT-PCR assays were designed to detect levels of equine clock genes. Expression profiles for *Per2*, *Bmal1* and *Cry1* were first determined in a synchronized equine cell line. Subsequently, expression in equine whole blood and adipose tissue was assessed. High amplitude circadian oscillations of *Per2*, *Bmal1*, and *Cry1* were observed *in vitro* in response to a synchronizing stimulus. A synchronized molecular clock was also demonstrated in equine adipose tissue although oscillation of *Bmal1* was less robust than that of *Per2* and *Cry1*. In contrast to previous studies in humans and rats however, there was no evidence of synchronized clock gene expression in equine peripheral blood. These studies suggest that synchronous control of clock gene oscillation in equine peripheral blood is not as tightly regulated as in other species and may reflect the influence of different evolutionary challenges modifying the function of a peripheral clock.

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### 3.2 Introduction

In order to align physiological function with the solar day, molecular clock mechanisms have evolved that are sensitive to light and allow mammals to anticipate periods of activity. The central pacemaker of the mammalian circadian timing system, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, receives light information via the retino-hypothalamic tract and transmits the timing signal to peripheral tissues (Perreau-Lenz *et al.* 2004;Yamazaki *et al.* 2000). This timing information serves to synchronize self-sustained independent circadian oscillators that are now thought to exist within each cell of almost every tissue (Nagoshi *et al.* 2004;Welsh *et al.* 2004;Yoo *et al.* 2004). In this way, peripheral tissues can adapt their specific function to the correct time of day by means of tissue-specific circadian regulation of transcription, as has been revealed by several microarray studies (Desai *et al.* 2004;Kita *et al.* 2002;Panda *et al.* 2002;Storch *et al.* 2002;Yamamoto *et al.* 2004;Zambon *et al.* 2003). In the absence of an entraining signal, cells in peripheral tissues become desynchronous and overall tissue oscillations dampen. Cell culture experiments have revealed that synchronicity can easily be restored by a number of methods (Balsalobre *et al.* 1998;Balsalobre *et al.* 2000).

The molecular clock within the SCN consists of gene-protein-gene feedback loops whereby the protein has a negative feedback effect on its own transcription and stimulates the transcription of other clock genes (Reppert and Weaver 2002). *Bmal1* transcription provides the positive driving force by binding to constitutively expressed *Clock* (Hogenesch *et al.* 1998). CLOCK-BMAL1 heterodimers bind to E-box motifs upstream of *Cry* (*cryptochrome*) and *Per* (*Period*) genes to initiate their transcription. PER and CRY relocate to the nucleus and interfere with CLOCK-BMAL1 DNA binding, providing the negative feedback loop (Kume *et al.* 1999;Shearman *et al.* 2000). Positive feedback is provided by PER2 contributing to the transcription of *Bmal1* (Yamamoto *et al.* 2004). Recent studies have identified a second feedback loop comprising the opposing activities of the *Rora* and *Rev-erb alpha* orphan nuclear receptors (Sato *et al.* 2004). The authors demonstrated that *Rora* acts as a transcriptional activator of *Bmal1* while *Rev-erb alpha* represses its

expression. This combination of loops, in conjunction with posttranslational mechanisms contributing to the time delays needed for a 24 hr clock (Reppert and Weaver 2002) ensures perpetuation of the self-sustaining nature of the molecular clock.

Initial investigations of peripheral clocks were confined to studies in small mammals, particularly nocturnal rodents. Results in these species have demonstrated that peripheral tissues share a similar temporal pattern of clock gene expression, exemplified by the antiphase oscillation of *Per2* and *Bmal1* mRNAs exhibited in heart, lung, liver, eye, kidney and pancreas (Andersson *et al.* 2005;Muhlbauer *et al.* 2004;Oishi *et al.* 1998b). While human studies are confined to less invasive measurements of clock gene expression in peripheral blood, oral mucosa and skin, similar temporal clock gene expression has been observed (Bjarnason *et al.* 2001;Boivin *et al.* 2003;Kusanagi *et al.* 2004). Recent results from *in vivo* studies in sheep have also demonstrated robust cycling of clock genes in the liver (Andersson *et al.* 2005). In contrast to sheep and rodents, the horse is unique in that it shares a physiological capacity for elite athleticism with the human. Globalization of equestrian competition and in particular the Thoroughbred racing industry means that the equine athlete, similar to its human counterpart, is frequently subject to the detrimental physiological effects associated with transmeridian travel. Elucidation of the molecular mechanisms of equine peripheral clocks will provide the groundwork for future studies on the consequences of jet lag in the horse, in addition to expanding the range of species used in circadian research studies and including a large diurnal mammal of significant economic importance.

The goal of the present study was to investigate clock gene expression in two equine peripheral tissues, specifically whole blood and adipose tissue. First, an *in vitro* model was used to investigate the mechanisms of an equine peripheral clock. A serum shock protocol using cultured fibroblasts has been employed extensively as a tool to unravel the complex feedback loops of the molecular clock (Allen *et al.* 2001;Balsalobre *et al.* 1998;Yagita *et al.* 2001). In addition, cell culture experiments have proven invaluable in determining the cell autonomous, self-sustainable nature of clock mechanisms and the role of the SCN as a synchronizer of peripheral rhythms

(Welsh *et al.* 2004). Using equine fibroblasts, we employed this technique to validate the efficiency and sensitivity of Real-Time reverse transcription (RT)-PCR assays designed to detect oscillating clock gene transcripts in the horse and to determine whether the mechanisms of an equine peripheral oscillator resemble those of the core oscillator in other species. As it is not practical to sacrifice a large mammal species such as the horse for the investigation of clock gene expression in the SCN or other internal organs, tissues were chosen that permit less invasive tissue collection and multiple sampling times from the same animal. Rhythmic cycling of clock genes was previously demonstrated in peripheral blood of rats (Oishi *et al.* 1998a) and humans (Teboul *et al.* 2005; Kusanagi *et al.* 2004; Boivin *et al.* 2003). Similarly, it has been determined that adipocytes possess the molecular machinery for a biological clock (Aoyagi *et al.* 2005). We therefore hypothesized that synchronized peripheral clocks would be detectable in these tissues in the horse. *Per2*, *Bmal1* and *Cry1* were selected for analysis as they are key components of the circadian clock and have been shown to exhibit robust oscillations in the peripheral tissues of other species.

### **3.3 Methods**

#### **3.3.1 Animals**

Four healthy, lean, 3 yr old mares of mixed breed, with body condition scores (BCS) (Henneke *et al.* 1983) ranging from 4-5 (on a scale of 1-9, 1 = very thin, 5 = normal, 9 = extremely fat), were randomly chosen from the research herd for use in peripheral blood monitoring. The group of mares used for investigation of adipose tissue ranged in age from 7 to 16 yrs, with BCS of 5-8. Older animals were used in this experiment as they had greater fat deposits and were easier to sample. Animals were maintained outdoors under conditions of natural photoperiod prior to each experiment. Several days prior to each experiment, mares were housed in individual stalls under a lighting schedule that mimicked the natural photoperiod for that time of year. The day before sampling began, mares were fitted with indwelling jugular catheters. Throughout the experiments, sampling during the hours of darkness was

conducted with the aid of only a dim red light from handheld flashlights. Blood samples were assayed for melatonin to ensure that animals were normally entrained to the light/dark cycle. Access to water was *ad libitum* and feed was provided 4 times a day to prevent a conspicuous 24-hour temporal cue (Piccione *et al.* 2002). All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC).

### ***3.3.2 Quantitative Real-Time Reverse-transcriptase polymerase chain reaction (RT-PCR)***

Equine *Per2*, *Bmal1* and *Cry1* cDNA sequences were isolated from a prepared equine lymphocyte cDNA library by polymerase chain reaction (PCR) as described in Chapter 2. RT-PCR primer sequences and target specific fluorescence-labeled Taqman probes (Biosearch Technologies, Novato, CA) were then designed using equine nucleotide sequence data for each gene (Table 3.1). Primers were intron spanning with the exception of *Per2*. Reverse transcription (RT) and amplification were performed using a Smart Cycler real time thermal cycler (Cepheid, Sunnyvale, CA). This system allows the detection of increasing amounts of amplicons at every PCR cycle. The efficiency of each primer/probe combination was tested by running serial 10-fold cDNA dilutions. The correlation between the Ct value (the number of PCR cycles required for the fluorescent signal to reach a threshold level) and the amount of cDNA standard was linear over a 5-log range for all assays. Each 25  $\mu$ l reaction contained 1 x EZ buffer (Applied Biosystems, Foster City, CA), 300  $\mu$ M of each dNTP, 2.5 mM manganese acetate, 200 nM forward and reverse primer, 125 nM fluorogenic probe, 40 U RNasin (Roche, Indianapolis, IN) and 2.5 U rTth (Applied Biosystems). Cepheid also recommends the addition of an 'Additive Reagent' to prevent binding of polymerases and nucleic acids to the reaction tubes. This reagent was added to give a final concentration of 0.2 mg/ml bovine serum albumin (non-acetylated), 0.15 M trehalose and 0.2 % Tween 20. Thermocycler parameters consisted of a 30-minute RT step at 60°C, 3 minutes at 94°C and 40 cycles of: 94°C

for 15 seconds (denaturation) and 60°C for 30 seconds (annealing and extension). In the case of each sample, quantitative measurement of the level of transcripts of the housekeeping gene product  $\beta$ -glucuronidase (*GUS*) was used as an internal control of sample-to-sample differences in RNA concentration. Expression levels of clock genes are reported as the number of transcripts per number of *GUS* molecules. *GUS* was first tested for its suitability as an endogenous control in equine peripheral blood and adipose tissue by confirming that its expression levels did not vary significantly across sampling times.

**Table 3.1** Taqman Primer/Probe combinations for Real-Time RT-PCR

Gene	Primer/Probe	Sequence (5'-3')
<i><math>\beta</math>-glucuronidase</i>	Forward	AAGAATATGTGGTTGGAGAGCTCATCT
	Reverse	CGCAAAGGAATGCTGCACCT
	Probe	ATGACTGACCAGTCACCGCAGAGAGCAATGGG
<i>Per2</i>	Forward	CCAGCAAATATTTTCGGAAGCATCGA
	Reverse	GCCATCAGCAGCCAGACAGG
	Probe	AGCGAAAGCGAAGGTGGACGTGGACGGAAG
<i>Bmal1</i>	Forward	CCACCAATCCATACACAGAAGCAAAC
	Reverse	TCTTCCCTCGGTCACATCCT
	Probe	CACCTCATTCTCAGGGCAGCAGATGGATTTTT GTTTGTCG
<i>Cry1</i>	Forward	CGGTTTGGGTGTCTGTCGTGTC
	Reverse	CGCAGATGGGGTTTCCTTCCATTTTATCA
	Probe	TGGGCAACTGTTATGGCGTGAATTTTCTACA CGGCAGCAAC

### ***3.3.3 Clock gene expression profiles in serum-shocked equine fibroblasts***

Equine fibroblasts derived from a diploid cell line (ATCC CCL-57) (dermis, equus caballus, 4-yr old quarter horse mare) were grown to confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum (Gibco, Grand Island, NY) and then maintained in DMEM containing 5 % fetal calf serum for 4 days prior to serum shock. Cells were changed to a medium containing 50 % adult horse serum and incubated for 2 hr, after which the serum-rich medium was replaced with serum-free medium. Cells were rinsed with cold phosphate buffered saline (PBS) and whole cell RNA isolation was carried out using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA) every 4 h for 52 h. *Per2*, *Bmal1* and *Cry1* mRNA levels were determined at each time point using quantitative Real-Time RT-PCR.

### ***3.3.4 Clock gene expression profiles in equine peripheral blood***

Beginning at 0700 h, 2.5 ml of blood was collected into PAX gene Blood RNA tubes (QIAGEN, Valencia, CA) at 4-h intervals for 48 h. Total RNA was isolated from each tube according to the PAX gene Blood RNA Kit recommendations. During RNA isolation, an additional on-column DNA digestion was performed with the RNase-Free DNase Set (QIAGEN) for quality assurance. Taqman quantitative Real-Time RT-PCR was performed using a Smart Cycler real-time thermal cycler (Cepheid) to determine the expression level of equine *Per2*, *Bmal1*, *Cry1* and *GUS*. A second 6 ml blood sample was taken at each time point, allowed to clot and kept overnight at 4°C. The next day, samples were centrifuged and the serum harvested and stored at -20°C until assayed for melatonin. This experiment was conducted at the time of year (late May) corresponding to a 15 h light/9 h dark (LD15:9) natural photoperiod (longitude W84.5, latitude N38.1).



### ***3.3.5 Clock gene expression profiles in equine adipose tissue***

Beginning at 1200 h and continuing at 2-h intervals for 24 h, 100 mg of adipose tissue was collected from the fat pad near the tail head region of each mare by a stab incision followed by a punch biopsy. Prior to each surgery, mares were sedated by administration (IV) of 10 mg Dormosedan® (Pfizer Animal Health, New York, NY) and 5 mg Torbogesic® (Wyeth, Madison, NJ). Samples were immediately snap frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (BIO-RAD, Hercules, CA) according to the manufacturer's instructions with two exceptions. First, following disruption of the tissue with a handheld rotor-stator homogenizer and cell lysis in PureZOL (BIO-RAD) reagent, an additional 10-minute centrifugation step was performed at 4°C. This is recommended for lysate from tissues rich in fat. Second, addition of 500 ul of chloroform to the lysate yielded greater RNA concentrations in preliminary tests using this kit for extraction of total RNA from equine adipose tissue. The protocol includes a DNase 1 digestion step to ensure removal of any contaminating DNA. A 6ml blood sample was also taken at each time point for melatonin analysis. This experiment was conducted at the time of year (late January) corresponding to a 10 h light/14 h dark (LD15:9) natural photoperiod (longitude W84.5, latitude N38.1).

### ***3.3.6 Melatonin Radioimmunoassay (RIA)***

Melatonin was measured by a commercial RIA kit (Alpco, Windham, NH) as described previously (Fitzgerald *et al.* 2000). Briefly, a 1 ml serum aliquot was extracted according to the directions of the manufacturer and reconstituted in a buffer solution provided. Aliquots of the extracted samples were assayed in duplicate. Inter- and intra-assay coefficients of variation for low melatonin concentration pool were 9.8% and 8.2% respectively. For the high concentration pool, the inter- and intra-assay coefficients of variation were 12.8% and 11.1% respectively. The limits of detection of the assays averaged 0.5 pg/ml.

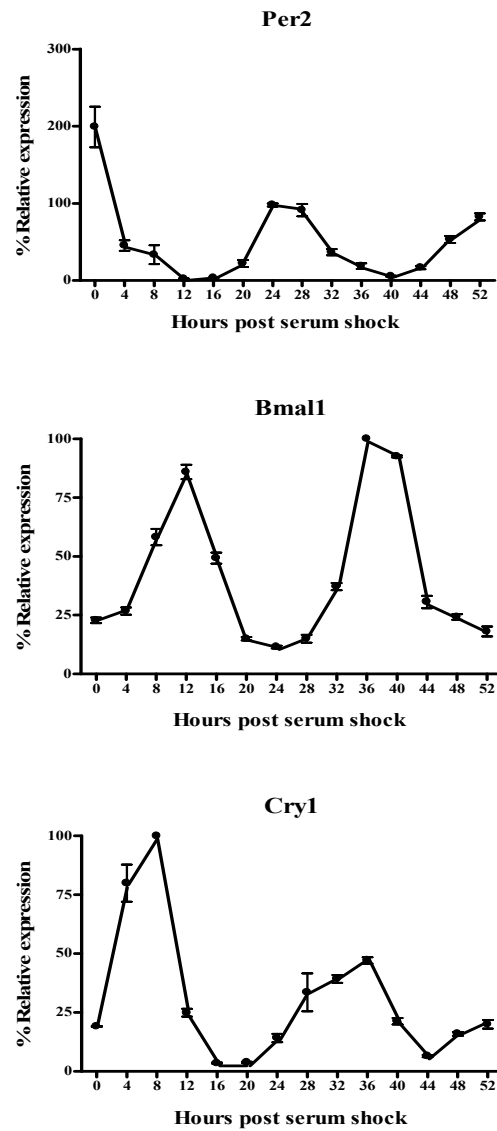
### **3.3.7 Statistical Analysis**

Daily variation of mRNA expression was statistically analyzed using repeated measures analysis of variance (ANOVA) with Graph Pad Prism Version 4.0 for Windows, Graph Pad software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). The values of the relative expression of mRNA are presented as the mean  $\pm$  SEM. A value of  $p < .05$  was considered significant.

## **3.4 Results**

### **3.4.1 Clock gene expression in equine fibroblasts**

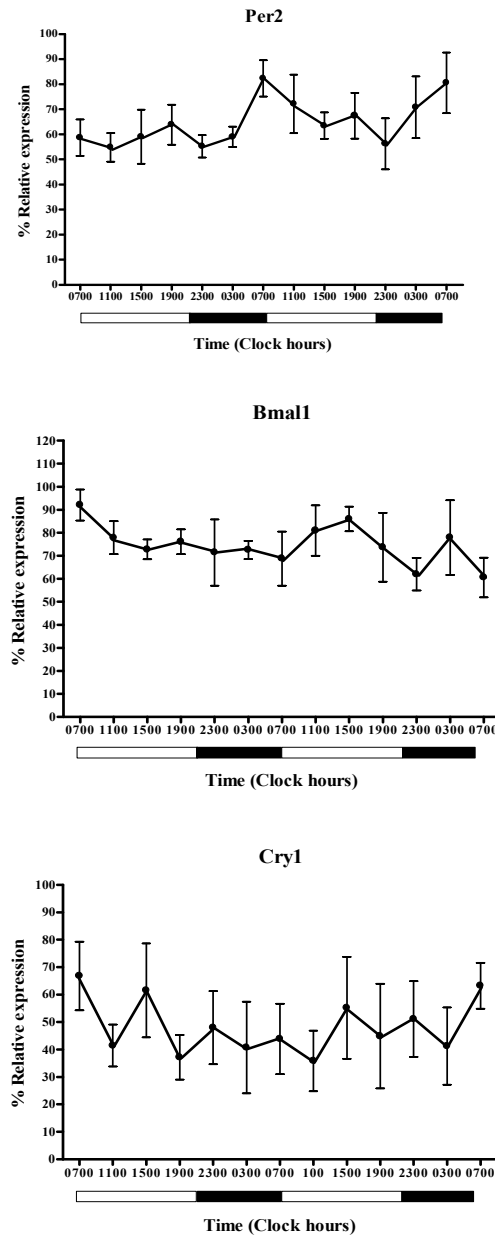
Repeated measures ANOVA ( $n = 4$ ) revealed a significant variation in expression levels over time for all three clock genes ( $p < .0001$ , respectively, Figure 3.1). Equine *Per2* was rapidly induced following serum shock to levels 90-fold greater than trough values. Expression levels then declined, before rising again 24 and 52 h later. *Bmal1* levels peaked antiphase to *Per2* at 12 and 36 h respectively, demonstrating an 8-fold peak-trough difference. *Cry1* peaked at 8 h post-serum shock, demonstrating a 29-fold increase from trough values, with a second peak at 36 h.



**Figure 3.1** Relative mRNA levels of equine *Per2*, *Bmal1* and *Cry1* in serum-shocked equine fibroblast cells over a 52-hour period. Each time point represents the mean  $\pm$  SEM for three separate experiments ( $n = 3$ ). Expression of all three clock genes demonstrated significant variation over time ( $p < .0001$ , repeated measures ANOVA).

### 3.4.2 Clock gene expression in equine peripheral blood

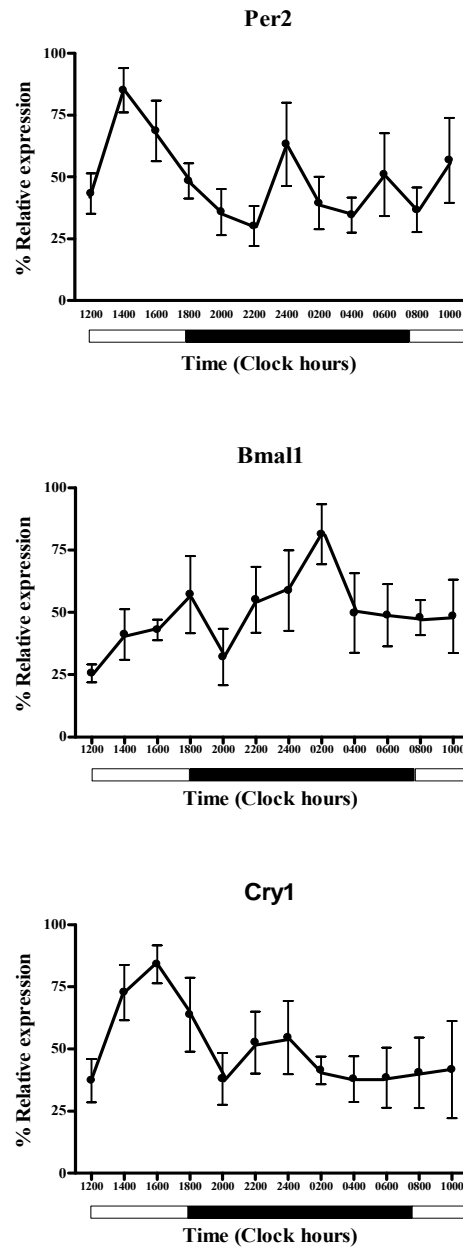
In contrast to the high amplitude cycling demonstrated in a synchronized equine cell line, no significant differences in daily expression of *Per2*, *Bmal1* and *Cry1* were detected in equine peripheral blood (Figure 3.2). Values for the control gene *GUS* also remained constant over time.



**Figure 3.2** Relative mRNA levels of equine *Per2*, *Bmal1* and *Cry1* in equine peripheral blood. The data is represented as the mean  $\pm$  SEM for four mares (n = 4). No significant daily variation was found in the expression of *Per2*, *Bmal1* and *Cry1* mRNA in peripheral blood. The white bars indicate the light period and the black bars indicate the dark period.

### ***3.4.3 Clock gene expression in equine adipose tissue***

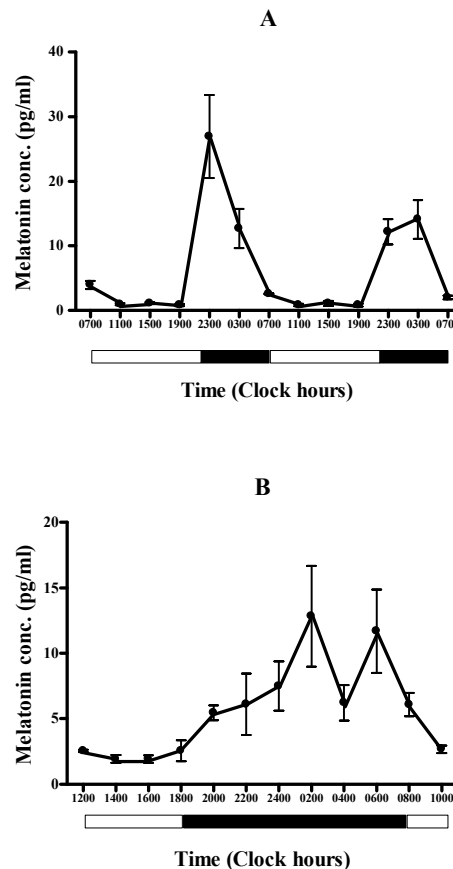
Both *Per2* and *Cry1* expression exhibited significant daily variation ( $p < .05$ ) in equine adipose tissue (Figure 3.3). Peak expression of *Per2* occurred at 1400 h, mid-way through the light phase, with a maximum peak-trough difference of 3-fold. *Cry1* reached maximal expression at 1600 h, 4 hours after the *Per2* peak, with a 4-fold peak-trough difference. *Bmal1* expression did not vary significantly over time. However, while the low level oscillation is below statistical significance using repeated measures ANOVA ( $p = .1354$ ), highest expression was observed at 0200 h, 12 h antiphase to the *Per2* peak, during the hours of darkness (Figure 3.3).



**Figure 3.3** Relative mRNA levels of equine *Per2*, *Bmal1* and *Cry1* in equine adipose tissue. The data is represented as the mean  $\pm$  SEM for four mares ( $n = 4$ ). Daily expression of *Per2* and *Cry1* mRNA varied significantly over time ( $p < .05$ , repeated measures ANOVA). The white bars indicate the light period and the black bars indicate the dark period.

#### 3.4.4 Daily variation of melatonin

During the 48 and 24-h experimental sampling periods all animals showed the expected daily variation in blood melatonin (Figure 3.4) reflecting the respective light/dark (LD) cycles for the time of year. Repeated measures ANOVA demonstrates significant differences over time in all experimental subjects ( $p < .0001$ ) with high values occurring during the hours of darkness and almost undetectable levels during daylight hours. These data indicate that the animals were normally entrained to the light/dark cycle during both experiments.



**Figure 3.4** Daily profiles of serum melatonin during (A) peripheral blood sampling and (B) adipose tissue sampling. The data is represented as the mean  $\pm$  SEM for four mares ( $n = 4$ ). Serum melatonin levels varied significantly over time ( $p < .0001$ ,

repeated measures ANOVA). The white bars indicate the light period and the black bars indicate the dark period.

### **3.5 Discussion**

#### ***3.5.1 Oscillating clock gene expression in vitro***

This study provides the first evidence of expression of the core molecular clock components *Per2*, *Bmal1* and *Cry1* in the horse. An *in vitro* model system was used to investigate the temporal pattern of clock gene cycling in an equine fibroblast cell line. In the absence of resetting stimuli or timing signals from the SCN, individual cell clocks gradually drift out of synchrony with each other. This dampening of circadian rhythms in peripheral cells and tissues is now understood to reflect a gradual desynchrony of many independent cellular oscillators (Welsh *et al.* 2004). Temporary resynchronization of these component oscillators occurs when cultured cells are stimulated by a number of different methods, most commonly by a change of culture medium to one containing a high serum concentration (Balsalobre *et al.* 1998; Balsalobre *et al.* 2000). For this reason, it has been suggested that fibroblasts may serve as a valid model for investigation of core circadian clock function (Rosbash 1998; Yagita *et al.* 2001). The current study clearly demonstrates rhythmic oscillations of equine clock genes in a fibroblast cell line following serum shock. Consistent with its role as an immediate early gene (Albrecht *et al.* 1997; Shearman *et al.* 1997), *Per2* was rapidly induced, before decreasing to minimal levels followed by a robust 24-h oscillation. As expected, *Bmal1* expression peaked 12 h after *Per2* with a subsequent inverse expression profile as has previously been reported (Oishi *et al.* 1998b). *Cry1* also demonstrated a robust circadian oscillation peaking 8 and 36 h post-serum shock. A similar expression pattern for *Cry1* was demonstrated in Rat-1 fibroblasts following synchronization by calcimycin (Balsalobre *et al.* 2000). These results confirmed the hypothesis that similar molecular clock mechanisms exist in an equine cell line as in the SCN and peripheral tissues of more commonly studied species. The *in vitro* model also served to validate



the Real-Time RT-PCR assays as highly sensitive and quantitative methods of detecting clock gene transcripts for subsequent *in vivo* experiments.

### ***3.5.2 Investigating clock gene expression in blood***

Evidence of clock gene oscillations in human (Boivin *et al.* 2003; Takata *et al.* 2002) and rat (Oishi *et al.* 1998a) peripheral blood cells led us to hypothesize that a synchronized molecular clock might also exist in equine peripheral blood. In marked contrast to the robust oscillations observed in serum-shocked fibroblasts however, clock gene expression did not vary over time in equine blood. Unlike other peripheral organs, blood is not a homogeneous tissue. Therefore, it might be reasoned that a failure to detect a rhythmic clock in whole blood is due to different temporal patterns of expression from a number of differentially synchronized cell types dampening the overall rhythm. However, similar profiles in *Per1* expression have been demonstrated in human peripheral mononuclear and polymorphonuclear cells, supporting the assumption that clock gene expression in different types of peripheral blood cells are entrained at the same phase angle (Kusanagi *et al.* 2004). The extent of regulation in this peripheral tissue is already the subject of scrutiny in other species. In a recent study using human subjects, highly variable inter-individual clock gene expression profiles were reported in peripheral blood mononuclear cells (Teboul *et al.* 2005). Two distinct molecular chronotypes were identified and the authors suggested that the circadian oscillator in the blood might be regulated differently from other known peripheral clocks. Communication between the SCN and peripheral tissues is thought to occur via both neural and humoral mechanisms (Allen *et al.* 2001; Guo *et al.* 2005; Terazono *et al.* 2003). One major difference in communication pathways between peripheral blood and other peripheral tissues is the absence of neural control through the autonomic nervous system. This lends further support to the idea that peripheral blood may be regulated differently by the SCN.

### 3.5.3 Investigation of clock gene expression in adipose tissue

Significant daily variation in *Per2* and *Cry1* mRNA expression was observed in equine adipose tissue. This is the first report of clock gene expression in this tissue in a large mammal. A similar temporal expression profile for these two gene transcripts has been reported previously in the SCN of mice (Kume *et al.* 1999) and sheep (Lincoln *et al.* 2002). The horses used in this experiment were sampled at the time of year corresponding to a 10 h light/14 h dark (LD 10:14) natural photoperiod, in contrast to the typical LD 12:12 light schedule commonly employed in circadian studies. This difference makes it difficult to directly compare temporal patterns of expression of these genes in the horse with peripheral tissues of animals entrained to alternative artificial light/dark cycles. For example, Lincoln and colleagues (2002) demonstrated markedly different phase relationships between *Per2* and *Cry1* expression in an ovine peripheral clock under a long day (LD 16:8) versus a short day (LD 8:16) photoperiod. Nevertheless, one common feature shared by both equine adipose tissue and ovine peripheral tissues under a short day photoperiod is the apparent inverse relationship between *Bmal1* and *Per2* peak expression (Andersson *et al.* 2005; Lincoln *et al.* 2002). Although statistically insignificant, highest expression of *Bmal1* was observed at 0200 h, 12 h after the peak in *Per2* expression.

Several factors may explain the reduced robustness of the *Bmal1* oscillation. In a previous study using mouse adipose tissue, it was determined that the expression phase of *Bmal1* was more advanced in adipocytes than in the stromal vascular fraction (Aoyagi *et al.* 2005). As the adipose tissue examined in this study was not fractionated, it is possible that the reduced robustness observed is a result of overlapping phases of temporal expression in the separate fractions. A common criticism of gene expression data from adipose tissue is difficulty in controlling for the presence of mononuclear leucocytes in the samples. However, the lack of clock gene oscillation observed in equine peripheral blood would suggest that this source of mononuclear cells does not contribute to the oscillating expression and may in fact contribute to reduced robustness.

Adipose tissue secretes a variety of biologically active molecules including leptin, resistin and adiponectin (Matsuzawa *et al.* 2004), many of which have now been shown to exhibit diurnal rhythms (Gavrila *et al.* 2003). In obese individuals, altered expression of these adipocytokines have been linked to the development of insulin resistance and metabolic syndrome (Arita *et al.* 1999; Matsuzawa *et al.* 2004; Stefan *et al.* 2002). Another study of clock gene expression in adipose tissue found that expression levels were significantly attenuated in obese mice. It was suggested that clock genes may function by regulating the expression of adipocytokines and that obesity may be the result of a dampening of this regulation (Ando *et al.* 2005). Since there was some variation in amounts of fat tissue between mares used in this experiment based on the range of BCS, it is also possible that different degrees of adiposity affected the overall robustness of clock gene expression. In addition, a neural connection between the SCN and white adipose tissue has been demonstrated in mice (Bamshad *et al.* 1998) as has the role of the SCN in lipid mobilization (Teixeira *et al.* 1973). All of the above indicate the importance of a functional molecular clock in adipose tissue metabolism. The current results suggest that a synchronized peripheral clock is present in equine adipose tissue, although this may be subject to variation between individuals based on degree of adiposity.

### ***3.5.4 Characteristics that may affect circadian regulation in the horse***

Food induced phase-resetting of peripheral clocks has been shown to occur in the liver, kidney, heart and pancreas of mice, with the greatest effect observed in the liver (Challet and Pevet 2003). Nocturnal rodents consume ~ 80% of their food during the hours of darkness. This contrasts with constant grazers such as the horse. It was tentatively suggested that ruminants such as sheep, which alternate their day between periods of foraging and ruminating, are less likely to be dependent on feeding cues for entrainment of their peripheral clocks (Andersson *et al.* 2005). It is

feasible that the same holds true for the horse. In a feral environment, horses disperse the approximate 15 h allocated to feeding behavior throughout the 24-h period.

An additional distinction between the horse and other species commonly used for investigation of biological clocks is their sleep-wake patterns. The sleep-wake cycle is one of the circadian rhythms that can be most readily perceived and defined in other species. For example, rodents such as mice and rats sleep an average of 12-13 h in every 24-h period (Campbell and Tobler 1984). Similarly, most healthy adult humans allocate 8 out of every 24 h for consolidated sleep (Campbell and Tobler 1984). In contrast, the horse only sleeps in short 15-minute bursts for an average of 2.9 h a day (Dallaire 1986). In addition, sleep periods are not confined to the hours of darkness. Prey animals are most vulnerable to predators during periods of rest. For this reason, small mammals often seek safe refuge from predators in underground burrows during these times. In the case of a large migratory animal such as the horse, that remains highly visible to predators when at rest, it is conceivable that circadian regulation of certain peripheral tissues may be less tightly regulated in this than in species of rodents. Recent studies of gene expression in rodents have revealed unique subsets of circadian regulated genes in individual peripheral tissues (Panda *et al.* 2002; Storch *et al.* 2002). This has led to the assumption that the function of peripheral clocks is to adaptively anticipate daily changes that might influence local physiology. One possible evolutionary explanation for the lack of a synchronized molecular clock in peripheral blood of the horse is that the horse must be able to adapt its physiology to react to danger at any point in the 24-h cycle.

In many respects, the horse is not unlike the common vole, *Microtus arvalis*. Both are hind-gut fermentors that disperse their feeding and activity rhythms throughout the 24-h period, in ultradian 2 to 3-h bursts in the case of the vole (Daan and Slopsema 1978; Gerkema *et al.* 1990). Voles only show circadian modulation of behavior when housed in cages and provided with running wheels (Gerkema *et al.* 1990) or a circadian feeding regimen (Van der Veen *et al.* 2006). Interestingly, in the absence of controlled circadian feeding and behavior regimens, such as their feral state, clock gene expression in vole peripheral tissues remains constant (Van der Veen *et al.* 2006), similar to our observations in equine blood under experimental

conditions that permit natural feeding behavior. These findings led Van der Veen and colleagues to suggest that animals that display a combination of ultradian and circadian behaviors may also display differences in the entrainment pathways for peripheral circadian clocks. In this regard, horses in their natural environment may also exhibit reduced circadian behavioral rhythmicity and this may explain the lack of synchronized clock gene oscillation in peripheral blood. While robust rhythmicity of many physiological variables has been documented in the horse (Piccione *et al.* 2005), these experiments were conducted under conditions where the animals were stabled and fed during times that reflected human diurnal activity rhythms. Important future experiments should investigate equine circadian rhythms under natural feral conditions and determine whether ultradian rhythms of equine behavior are revealed.

In summary, the oscillating rhythms of *Per2*, *Bmal1* and *Cry1* expression have been characterized in equine fibroblasts *in vitro* and in adipose tissue *in vivo*. In addition, no evidence was found of oscillating clock gene expression in equine peripheral blood. The horse is the first species studied in which there would appear to be a lack of a functioning clock in this tissue. While this result raises more questions than it answers, it encourages further investigation of peripheral clocks in large mammals. The absence of rhythmic expression of clock genes in equine whole blood may reflect a reduced dependency on time cues for regulation of at least one peripheral tissue in this species. The study highlights the importance of broadening the diversity of species used for investigation of circadian clocks. Additional studies in large mammals should further our understanding of the function of peripheral clocks in species representing the outcome of different evolutionary challenges.

## CHAPTER 4

### **Acute Systemic Inflammation Transiently Synchronizes Clock Gene Expression in Equine Peripheral Blood**

#### **4.1 Summary**

Peripheral clocks receive timing signals from the master mammalian pacemaker in the suprachiasmatic nucleus (SCN) and function to adaptively anticipate daily changes that influence local physiology. Evidence suggests that peripheral immune activation may act as a resetting signal for circadian clocks in peripheral tissues. This study investigated whether acute systemic inflammation could synchronize clock gene expression in equine peripheral blood, a tissue that does not normally oscillate in this species. In vivo administration of lipopolysaccharide (LPS) resulted in significant upregulation of the core clock genes *Per2* and *Bmal1* in peripheral blood, in association with an acute rise in *tumor necrosis factor (TNF)  $\alpha$*  and core body temperature compared to vehicle-treated control animals. Furthermore, co-administration of LPS and phenylbutazone, a non-steroidal anti-inflammatory drug (NSAID) known to inhibit prostaglandin (PG) E<sub>2</sub> synthesis in the horse, prevents both the febrile response and the synchronized increase in clock gene expression. However, the rise in *Per2* and *Bmal1* expression cannot be replicated in equine peripheral blood cells ex vivo by treatment with PGE<sub>2</sub>, LPS or a heat shock mimicking the in vivo febrile response. These results suggest an indirect communication pathway between immune modulators and the molecular machinery of cell clocks in peripheral blood. This potential immune feedback regulation of an equine peripheral clock implies a role for the circadian system in contributing to innate immune reactions and maintaining homeostasis in a tissue that acts as the first line of defense during an infectious challenge.

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## 4.2 Introduction

The mammalian circadian system, consisting of the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral oscillators located throughout the organism, permits physiological anticipation of environmental and internal changes by entraining biological phenomena to a 24-h cycle. The primary external zeitgeber is the photic input that travels from the eyes to the SCN via the retino-hypothalamic tract. Here, the light signal drives cellular machinery controlling the expression of clock genes that form autoregulatory transcription-translation feedback loops (Reppert and Weaver 2002). The core cellular oscillators within the SCN ultimately generate circadian rhythms in physiology and behavior by coordinating oscillations of peripheral clocks through neural and endocrine pathways (Balsalobre *et al.* 2000; McNamara *et al.* 2001; Terazono *et al.* 2003).

Rhythmic expression of the core molecular clock components *Per* (*Per1*, *Per2* and *Per3*), *Cry* (*Cry1* and *Cry2*), *Clock* and *Bmal1*, control circadian rhythms at the cellular level. The positive driving force is provided by BMAL1 heterodimerizing with constitutively expressed CLOCK to induce transcription of *Cry* and *Per* genes (Hogenesch *et al.* 1998). PER and CRY subsequently relocate to the nucleus and interfere with CLOCK-BMAL1 DNA binding providing the negative feedback loop (Kume *et al.* 1999; Shearman *et al.* 2000). PER2 also contributes to transcription of *Bmal1* providing positive feedback (Yamamoto *et al.* 2004). This basic oscillatory mechanism is supported by additional feedback loops (Preitner *et al.* 2002; Sato *et al.* 2004) and ensures perpetuation of the self-sustaining nature of the molecular clock.

Independent circadian oscillators exist within each cell of almost every tissue. (Nagoshi *et al.* 2004; Welsh *et al.* 2004; Yoo *et al.* 2004). Peripheral clocks share a similar temporal pattern of clock gene expression as the master clock in the SCN and synchronized clock gene expression has been reported in almost all tissues examined (Andersson *et al.* 2005; Muhlbauer *et al.* 2004; Oishi *et al.* 1998b), including peripheral blood cells of humans (Boivin *et al.* 2003; Kusanagi *et al.* 2004) and rats (Oishi *et al.* 1998a). Synchronizing signals from the SCN are thought to allow peripheral tissues to adapt their specific function to the correct time of day by means

of tissue specific circadian regulation of transcription (Desai *et al.* 2004; Kita *et al.* 2002; Panda *et al.* 2002; Storch *et al.* 2002; Yamamoto *et al.* 2004; Zambon *et al.* 2003). In peripheral blood, circadian rhythmicity is manifested in terms of clock gene expression, circulating levels and function of different cell types (Born *et al.* 1997; Kusanagi *et al.* 2004), cytokine production (Petrovsky *et al.* 1998) and natural killer cell function (Arjona and Sarkar 2006). However, we recently demonstrated that in contrast to all other species examined to date, clock gene expression in blood of healthy horses is not synchronized (Murphy *et al.* 2006). At the present time the reason for this is not fully understood but may reflect the influence of different evolutionary challenges modifying the function of this peripheral clock.

In addition to signals emanating from the SCN, clock gene expression in mouse peripheral tissues can also be entrained by pro-inflammatory stimuli (Marpegan *et al.* 2005). Significantly, PGE<sub>2</sub>, the primary mediator of endotoxic fever (Petrovsky and Harrison 1997), acts as a phase-resetting agent for mouse peripheral clocks *in vivo* and as a synchronizer of clock gene expression in cell cultures (Tsuchiya *et al.* 2005). A relationship between clock gene transcription and activation of innate immune responses is potentially highly significant as it raises important questions about control of the mammalian immune system. It is thought that the circadian system provides a temporal frame necessary to maintain physiological homeostasis (Matsunaga *et al.* 2000). The novel finding of an unsynchronized clock in equine peripheral blood prompted the current studies to investigate peripheral clock gene expression under physiological conditions that challenged the normal internal homeostasis in this tissue.

Based on the recent evidence that PGE<sub>2</sub> acts as a peripheral clock resetting agent in the mouse model (Tsuchiya *et al.* 2005) and that increased circulating levels of PGE<sub>2</sub> are observed simultaneously with the onset of fever (Davidson *et al.* 2001; Rotondo *et al.* 1988), this study investigated whether induction of acute systemic inflammation via peripheral administration of lipopolysaccharide (LPS) would synchronize clock gene expression in equine blood. Additionally, it was hypothesized that co-administration of LPS and a non-steroidal anti-inflammatory drug (NSAID), phenylbutazone, that has previously been demonstrated to inhibit



PGE<sub>2</sub> synthesis in the horse (Beretta *et al.* 2005), would block synchronization of clock gene expression. Furthermore, the study aimed to ascertain whether treatment of equine peripheral blood mononuclear cells (PBMCs) with LPS, PGE<sub>2</sub> or a heat shock could directly synchronize clock gene expression *ex vivo*.

## **4.3 Methods**

### **4.3.1 Animals**

Eight healthy mares (*Equus caballus*) of mixed light horse breed ranging in age from 3-4 years were used in this study. Animals were maintained outdoors under conditions of natural photoperiod prior to each experiment. Several days prior to each experiment, mares were housed in individual stalls under a lighting schedule that mimicked the natural photoperiod for that time of year. During the daylight hours stalls were lit by two 200 W light bulbs together with natural light from large windows in each stall. The average light intensity was 1000 lux in each stall throughout the day. While dawn and dusk were not artificially simulated using gradually increasing and decreasing light intensities, the windows allowed the horses to experience the actual gradual changes in natural light. The day before initiation of blood collection, mares were fitted with indwelling jugular catheters. Throughout the experiments, sampling during the hours of darkness was conducted with the aid of only a dim red light from handheld flashlights. Circulating concentrations of melatonin were determined by radioimmunoassay to ensure that animals were normally entrained to the light/dark cycle. Access to water was *ad libitum* and feed was provided 4 times a day to prevent a conspicuous 24-h temporal cue (Piccione *et al.* 2002). All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC).

#### ***4.3.2 Investigation of clock gene expression in equine peripheral blood following administration of LPS and co-administration of LPS plus phenylbutazone (PBZ)***

Beginning at dawn (circadian time (CT) 0 = lights-on) a baseline blood sample and core body temperature reading was collected from all animals followed by intravenous (i.v.) administration of either vehicle ( $n = 4$ ) or .045 $\mu$ g/kg LPS ( $n = 4$ ) from *Escherichia coli* 055:B5 (Sigma, Saint Louis, Missouri). A blood sample (2.5 ml) was collected into PAX gene Blood RNA tubes (Qiagen, Valencia, CA) every 2 h for the first 6 h post administration and then at 4-h intervals for a total of 22 h. Total RNA was isolated from each tube according to the PAX gene Blood RNA Kit recommendations. During RNA isolation, an additional on-column DNA digestion was performed with the RNase-Free DNase Set (Qiagen) for quality assurance. Total RNA was quantitatively assessed using the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (Agilent Technologies, Palo Alto, CA). Real-time RT-PCR was performed to determine the expression levels of equine *Per2*, *Bmal1*, *TNF $\alpha$*  and  *$\beta$ -glucuronidase (GUS)*. A second 6 ml blood sample was collected at 4-h intervals for 34 h, allowed to clot and kept overnight at 4°C. The next day, samples were centrifuged and the serum harvested and stored at -20°C until assayed for melatonin. Body temperature readings were determined per rectum hourly for the first 6 hours and then at 4 hr intervals for a total of 34 h. Blood samples for melatonin analysis and body temperature readings were collected for a longer time duration to determine whether treatment modified these robust phase markers of the biological clock. This experiment was conducted at the time of year (mid-September) corresponding to a 12-h light/12-h dark (LD12:12) natural photoperiod (longitude W84.5, latitude N38.1).

Following a six month interval, the four mares that received LPS were used in a second study to investigate the effect of co-administration of LPS and PBZ. Following collection of baseline samples at dawn (CT 0 = lights-on), 4.4 mg/kg phenylbutazone was administered i.v. 30 min prior to LPS (.045  $\mu$ g/kg) administration. A second phenylbutazone dose was administered after a 12-h interval. Blood samples were collected and body temperature determined at 2-h intervals for

the first 6 h and at 4-h intervals thereafter, for a total of 22 h. Real-Time RT-PCR assays were performed to determine the expression level of equine *Per2*, *Bmal1*, *TNF $\alpha$*  and  *$\beta$ -glucuronidase (GUS)*. This study was conducted at the time of year (late-March) corresponding to an approximate 12-h light/12-h dark (LD12:12) natural photoperiod (longitude W84.5, latitude N38.1).

#### **4.3.3 Real Time RT-PCR**

Equine *Per2*, *Bmal1*, *TNF $\alpha$*  and *GUS* cDNA sequences were identified, isolated and Taqman primers and probes designed as previously described (Murphy *et al.* 2006) with the exception of *TNF $\alpha$* . The relevant *TNF $\alpha$*  sequences are: forward primer 5'-cagtcaatcaaccctctggcccaga- 3'; reverse primer 5'-cggcttgigggttgctacaacatg-3'; probe 5'tcgaaccccaagtgacaagcctgtagc-3'. Taqman quantitative Real-Time RT-PCR was performed as described in Chapter 3

#### **4.3.4 Melatonin RIA**

Melatonin was measured by a commercial RIA kit (Alpco, Windham, NH) as described previously (Fitzgerald *et al.* 2000). Briefly, a 1 ml serum aliquot was extracted according to the directions of the manufacturer and reconstituted in a buffer solution provided. Aliquots of the extracted samples were assayed in duplicate. Intra-assay coefficients of variation for low and high melatonin concentration pools were 14% and 5% respectively. The limits of detection of the assay averaged .5 pg/ml.

#### **4.3.5 Peripheral blood mononuclear cell (PBMC) culture**

Peripheral blood samples were collected into heparinized tubes via jugular venipuncture from a young, healthy horse and PBMCs were isolated from the buffy coat following centrifugation on Ficoll-Paque (GE Healthcare, Sweden). Cells were washed twice with Ca<sup>+2</sup> and Mg<sup>+2</sup>- free phosphate buffered saline (Sigma) and

resuspended at a final concentration of  $6 \times 10^6$  cells/ml in RPMI 1640 media (Gibco, Invitrogen corporation, Grand Island, NY) supplemented with 2.5 % autologous serum, 100 U/ml streptomycin/penicillin and 2 mM L-glutamine (Sigma). Cells were incubated in suspension on a rotator for 48 h at 37 °C in a 5 % CO<sub>2</sub>/air mixture prior to each experiment. Cell viability was determined on a Vicell™XR Cell Viability Analyzer (Beckman Coulter, Fullerton, CA).

PBMC cell cultures were treated with 1 µg/ml LPS to assess the possibility that LPS acts directly on blood cells to synchronize clock gene expression. Total RNA was isolated using the RNeasy® mini kit (Qiagen) at 2-h intervals for 8 h and again at 24 h post-treatment. mRNA levels for *Per2* and *Bmal1* were determined by Real-Time RT-PCR and compared to untreated control levels at each time point. Additionally, *TNFα* mRNA levels were determined for the first four time points to act as a marker of PBMC activation in response to LPS.

To determine whether PGE<sub>2</sub> (1mM in PBS, Sigma) or a serum shock could directly synchronize PBMCs ex vivo, PGE<sub>2</sub> was added to the culture medium at a concentration of 100nM, 1µM and 10µM, or cells were changed to a medium containing 50 % adult horse serum and incubated for 2 h, after which the serum-rich medium was replaced with serum-free medium. Total RNA was isolated at 1 h, 12 h and 24 h post-treatment with PGE<sub>2</sub> or serum shock and *Per2* levels were compared to untreated control levels at each time point.

Finally, to investigate whether heat shock alone could synchronize clock gene expression in blood cells, PBMCs were transferred to an incubator set at 40 °C for 4 h, after which time they were returned to 37 °C. Control samples were held at 37 °C for the duration. Total RNA was isolated at 2-h intervals from the time of heat shock for a total of 8 h. Again, mRNA levels for *Per2* and *Bmal1* were determined by Real Time RT-PCR at each time point.

#### **4.3.6 Statistical Analysis**

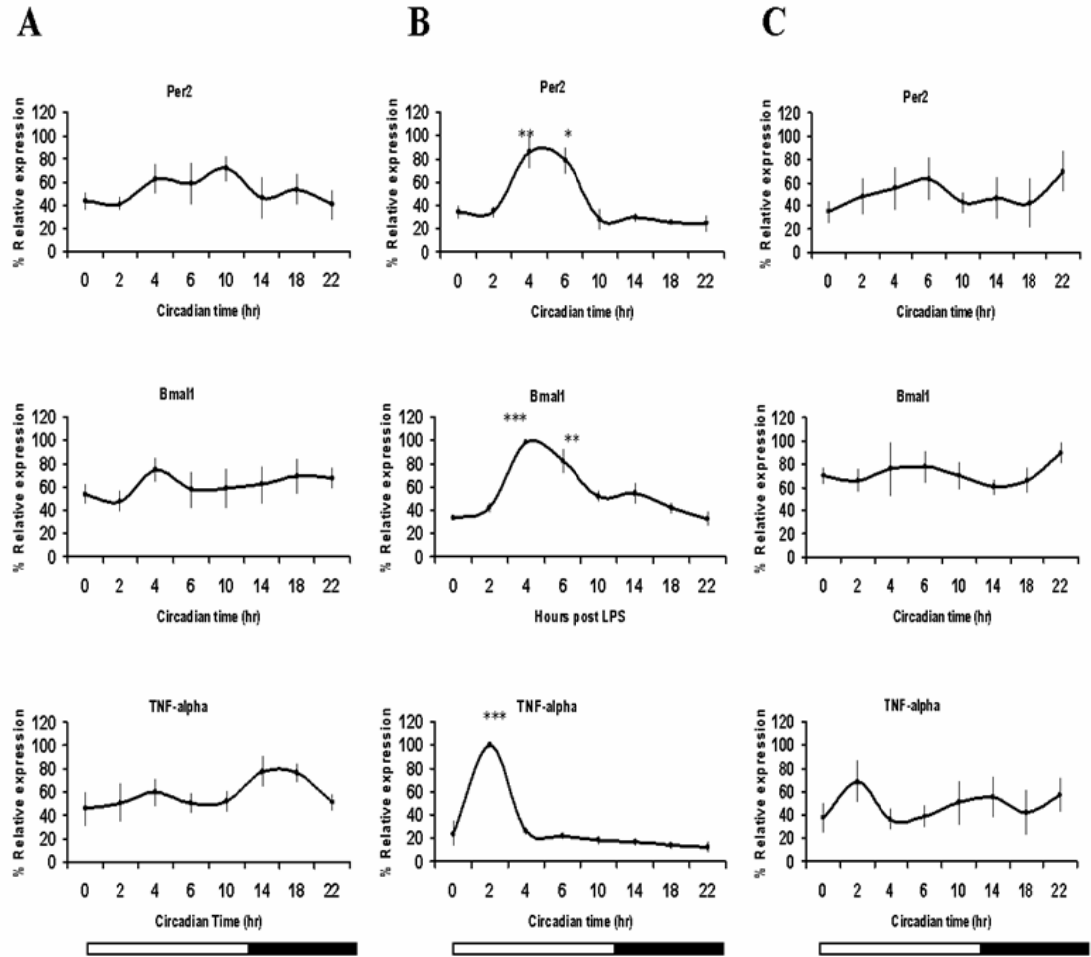
One-way repeated-measures analysis of variance (ANOVA) was used to assess variation in gene expression over time in the in vivo studies. Group differences were determined by two-way (time x treatment) repeated-measures ANOVA followed by Bonferroni post hoc tests where appropriate. In vitro results were analyzed using one- and two-way ANOVAs again followed by Bonferroni post hoc tests where appropriate. Data was analyzed using Graph Pad Prism Version 4.0 for Windows (Graph Pad Software, San Diego, CA,) and are presented as the mean  $\pm$  SEM. A value of  $p < .05$  was considered significant.

#### **4.4. Results**

##### **4.4.1 *Per2*, *Bmal1* and *TNF $\alpha$* expression profiles in peripheral blood of vehicle, LPS, and LPS plus phenylbutazone (PBZ) treated mares**

As previously demonstrated (Murphy *et al.* 2006), *Per2* and *Bmal1* mRNA expression did not vary over time in peripheral blood of control animals (Figure 4.1A). Similarly, expression levels of *TNF $\alpha$*  remained constant over the sampling period in vehicle-treated mares (Figure 4.1A). However, LPS administration produced a significant upregulation in *Per2* ( $F(7,3) = 8.12, p < .0001$ ), *Bmal1* ( $F(7,3) = 16.43, p < .0001$ ) and *TNF $\alpha$*  ( $F(7,3) = 46.82, p < .0001$ ) expression levels over time (Figure 4.1B) in equine peripheral blood. Peak expression of *Per2* occurred between CT4 and CT6 and post-tests revealed that expression levels at these time points were significantly different from all other time points ( $p < .01$  and  $p < .05$ , respectively). Peak expression of *Bmal1* coincided with those of *Per2* and again expression levels at CT4 and CT6 were significantly different from all other time points ( $p < .001$  and  $p < .01$ , respectively). Both *Per2* and *Bmal1* expression increased 3-fold from baseline values at these time points. Expression of *TNF $\alpha$*  increased 8-fold at CT2 and was significantly different from all other time points ( $p < .001$ ). Co-administration of LPS and PBZ abolished the upregulation in gene expression observed when LPS was

administered alone resulting in a lack of variation over time for all three genes (Figure 4.1C).

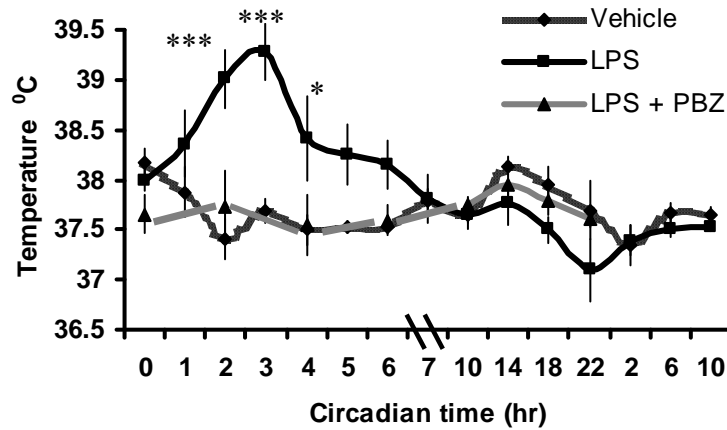


**Figure 4.1** Daily variation in mRNA expression levels of *Per2*, *Bmal1* and *TNF $\alpha$*  in peripheral blood of mares treated with (A) vehicle, (B) LPS, and (C) LPS plus PBZ. Total RNA was isolated from whole blood at circadian times (CT) 0, 2, 4, 6, 10, 14, 18 and 22. These time points correspond with 7am, 9am, 11am, 1pm, 5pm, 9pm, 1am and 5am, respectively. Vehicle or LPS were administered i.v. following the baseline sample at CT 0. PBZ was administered 30 min prior to LPS and again after a 12-h interval. Gene expression levels are expressed as the number of transcripts per number of molecules of the internal control gene *GUS* and are reported as a

percentage of the peak value. The data are represented as the mean  $\pm$  SEM ( $n = 4$ ) per time point. \*, \*\*, \*\*\* denote significant difference ( $p < .05$ ,  $p < .01$ ,  $p < .001$ , respectively) from values at all other time points. The white bars indicate the light period and black bars indicate the dark period.

#### ***4.4.2 Core body temperature changes in vehicle, LPS, and LPS plus PBZ treated mares***

Changes in body temperature over a 34-h period between vehicle and LPS-treated mares are shown in Figure 4.2. There was a significant time  $\times$  treatment interaction ( $F(14, 1) = 8.5$ ,  $p < .0001$ ) and a significant effect of time ( $F(14) = 6.66$ ,  $p < .0001$ ). The time  $\times$  treatment interaction was most apparent in the significant rise in body temperature in LPS-treated mares in the hours directly following LPS administration. Post hoc tests revealed significant differences between groups at CT2 ( $p < .001$ ), CT3 ( $p < .001$ ) and CT4 ( $p < .05$ ), respectively. While there appears to be a distinct disruption in the subsequent 24-h temperature rhythm (CT10-CT10; Figure 4.2) post-treatment, body temperature was not statistically different between groups at these time points. In addition to the induction of a febrile response, mild sickness behavior characterized by anorexia, lethargy and shivering was observed in LPS treated mares and subsided with a return to normal body temperature. In contrast to the acute temperature rise in LPS treated mares, there was no variation in body temperature or behavior of mares co-treated with LPS plus PBZ over a 22-h period.

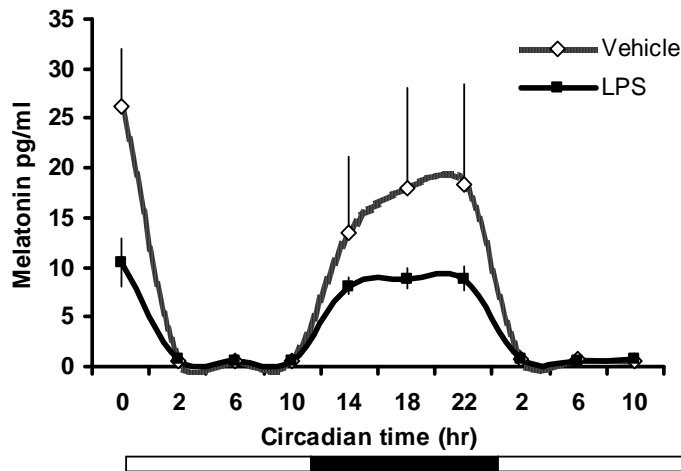


**Figure 4.2** Variation in core body temperatures over a 34-h period following i.v. administration of vehicle or LPS at CT0 and over a 22-h period for mares co-treated with LPS plus PBZ. Body temperature readings were taken hourly for the first 7 h, followed by readings at 4-h intervals. The data are represented as the mean  $\pm$  SEM ( $n = 4$ ) per time point. \*, \*\*\* denote significant difference ( $p < .05$  and  $p < .001$ , respectively) from values at all other time points.

#### 4.4.3 Daily variation in serum melatonin

There was no significant time  $\times$  treatment interaction, or treatment effect on serum melatonin levels between vehicle and LPS treated mares over the 34-h sampling period. However, the expected variation in blood melatonin reflecting the entraining light/dark cycle was apparent in the significant effect of time ( $F(9) = 9.9$ ,  $p < .0001$ ; Figure 4.3). High melatonin values occurred during the hours of darkness and levels were almost undetectable during daylight hours. These data indicate that the animals were normally entrained to the light/dark cycle during the study.

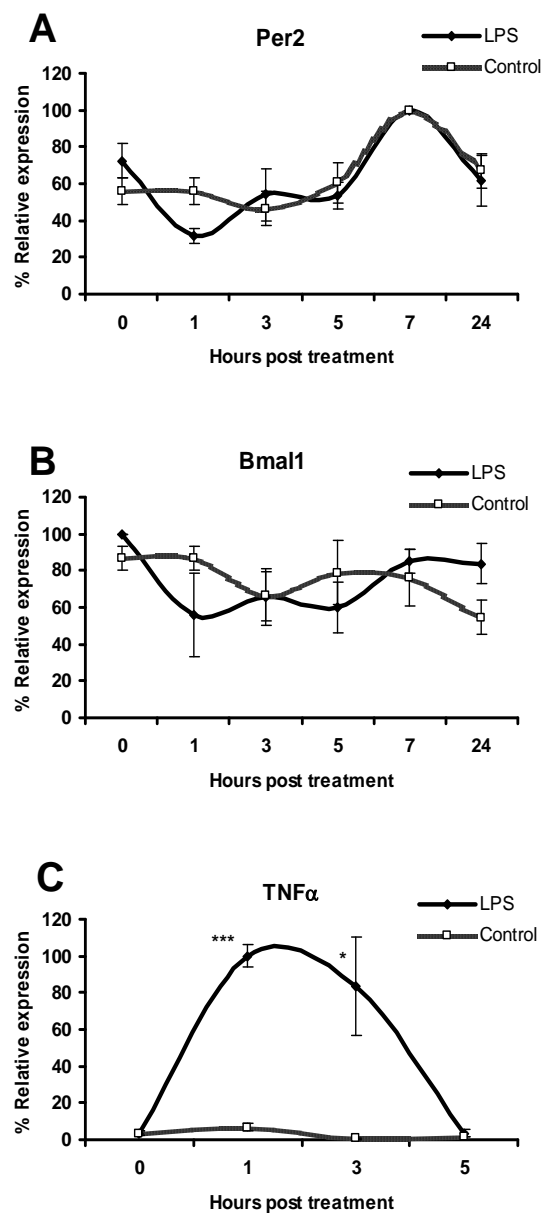




**Figure 4.3** Serum melatonin profiles over a 34-h period following i.v. administration of vehicle or LPS at CT 0. The data are represented as the mean  $\pm$  SEM ( $n = 4$ ) per time point. Melatonin levels varied significantly ( $p < .0001$ ) over time in all mares. The white bar indicates the light period and the black bar indicates the dark period.

#### 4.4.4 Gene expression in LPS stimulated PBMCs ex vivo

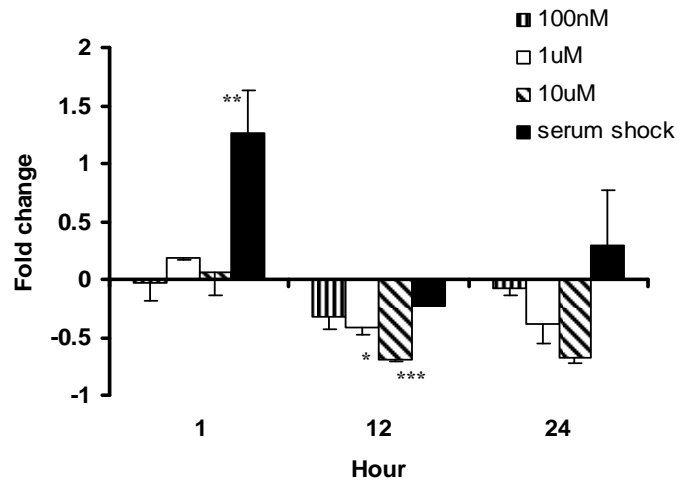
*Per2*, *Bmal1* and *TNF $\alpha$*  mRNA expression profiles were investigated following LPS stimulation and compared to non-stimulated control samples in ex vivo cultured PBMCs. There was no significant time x treatment interaction, or treatment effect on *Per2* expression between groups. However, a significant effect of time ( $F(5) = 9.59$ ,  $p < .0001$ ) on *Per2* levels was observed (Figure 4.4A). There was no time x treatment interaction, time effect or treatment effect on *Bmal1* expression (Figure 4.4B). In contrast, a significant time x treatment interaction ( $F(3,1) = 8.03$ ,  $p < .001$ ) plus an effect of time ( $F(3) = 8.91$ ,  $p < .001$ ) and treatment ( $F(1) = 26.5$ ,  $p < .001$ ) was observed for *TNF $\alpha$*  expression levels between groups (Figure 4.4C).



**Figure 4.4** mRNA expression of (A) *Per2*, (B) *Bmal1* and (C) *TNFα* in LPS stimulated and unstimulated PBMCs ex vivo. The data are represented as the mean  $\pm$  SEM of three independent experiments. \*, \*\*\* denote significant difference ( $p < .05$ ,  $p < .001$ , respectively) from values at all other time points.

#### 4.4.5 Effect of PGE<sub>2</sub> or serum shock on *Per2* expression in PBMCs ex vivo

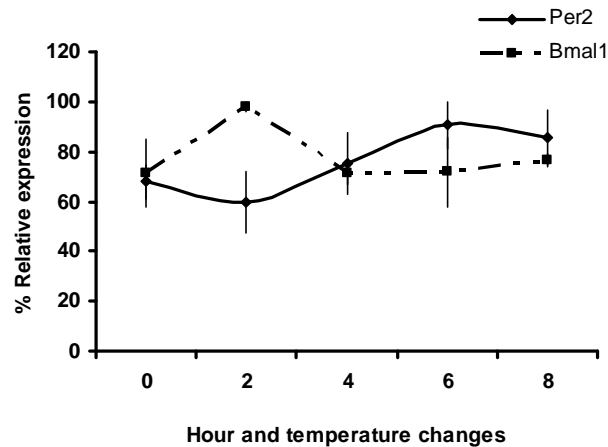
There was a significant time x treatment interaction ( $F(8,2) = 2.47, p < .05$ ) and a significant overall effect of time ( $F(2) = 4.97, p < .01$ ) and treatment ( $F(4) = 10.22, p < .0001$ ) on *Per2* expression. Post hoc tests revealed that only the serum shock procedure resulted in a significant increase in *Per2* expression 1 h post-treatment ( $p < .01$ , Figure 5). None of the PGE<sub>2</sub> doses elicited a response at this time point. In fact, the 1  $\mu$ M and 10  $\mu$ M PGE<sub>2</sub> doses significantly repressed *Per2* expression compared to control values at 12 h ( $p < .05$  and  $p < .001$ , respectively) and levels were also reduced 24 h post-treatment (Figure 4.5).



**Figure 4.5** Fold changes in *Per2* expression from cultured PBMCs following treatment with 100 nM, 1  $\mu$ M and 10  $\mu$ M PGE<sub>2</sub>, or a 2-h serum shock in media containing 50% adult horse serum. Data are represented as the fold expression change from mean control levels of three independent experiments at each time point and are represented as the mean  $\pm$  SEM. \*, \*\*, \*\*\* denotes significant difference ( $p < .05$ ,  $p < .01$ ,  $p < .001$ , respectively) from values at all other time points.

#### 4.4.6 Effect of a heat shock on clock gene expression in PBMCs ex vivo

Transfer of PBMCs previously cultured at 37 °C to an incubator set at 40 °C for duration of 4 h had no effect on *Per2* and *Bmal1* gene expression over time (Figure 4.6).



**Figure 4.6** Changes in *Per2* and *Bmal1* gene expression in cultured PBMCs during and after a 4-h incubation period at 40 °C. The data are represented as the mean  $\pm$  SEM of three independent experiments.

### 4.5 Discussion

#### 4.5.1 LPS transiently synchronizes clock gene expression in equine blood in vivo

In this study, we investigated whether induction of acute systemic inflammation in the horse could have a synchronizing effect on core components of the molecular clock in peripheral blood, a tissue which does not demonstrate synchronized rhythmicity in expression levels of these genes under normal circumstances (Murphy *et al.* 2006). We report successful induction of systemic inflammation following peripheral administration of the endotoxin LPS, as confirmed

by a rapid rise in body temperature and circulating expression levels of the pro-inflammatory cytokine, TNF $\alpha$ , and the visible appearance of sickness behavior in the LPS- treated mares compared to vehicle- treated controls. Significantly, activation of the innate immune response resulted in the upregulation of *Per2* and *Bmal1* expression in peripheral blood following LPS treatment. This is the first report of an interaction between the circadian and immune systems in the horse and the first to show an effect of immune activation on clock gene expression in peripheral blood in any species studied to date.

The absence of rhythmic expression of clock genes in whole blood of healthy horses is thought to reflect a reduced dependency on time cues for regulation of this peripheral tissue in this species (Murphy *et al.* 2006). We observed a simultaneous rise in both genes in response to LPS whereas an anti-phase oscillatory relationship has previously been observed in peripheral tissues of other species under non-inflammatory conditions (Andersson *et al.* 2005; Muhlbauer *et al.* 2004; Oishi *et al.* 1998b). However, there have been conflicting reports on the temporal expression relationship between *Per2* and *Bmal1* in blood cells from humans (Boivin *et al.* 2003; Takata *et al.* 2002; Teboul *et al.* 2005). Teboul *et al.* (2005) report a highly variable daily pattern of *Per2* and *Bmal1* expression and reveal two distinct molecular chronotypes, one of which demonstrates these genes oscillating in phase with each other in this tissue. The absence of a neural communication pathway between the SCN and peripheral blood is one possible explanation for the variation in regulation of this tissue by the circadian system. Of the four mares treated with LPS in this study, one mare displayed a second peak in *Per2* and *Bmal1* expression 18 h following the initial rise (unpublished result), suggesting that rhythmicity of clock gene expression had been stimulated. It is possible that individual variation in response to endotoxin may have resulted in a dampened response in the other mares and may have become more evident at a higher dose of LPS.

The physiological function of *Per2* and *Bmal1* upregulation during acute systemic inflammation remains to be elucidated, but could be an integral component of the reactive homeostatic response to an immune challenge. A recent report demonstrates the involvement of *Bmal1*, in association with its heterodimerization

partner *Clock*, in chromatin remodeling (Doi *et al.* 2006), a key process in the control of a large array of nuclear transcriptional activities. It is conceivable that the rise in clock gene expression may be associated with a new role for the molecular clock in immune cell activation via chromatin remodeling, permitting the large scale gene changes observed in blood cells post-endotoxin challenge (Fannin *et al.* 2005). Fannin *et al.* (2005) demonstrated differential gene expression in whole blood collected at 2 h and 6 h post-LPS treatment in rats and could discriminate between subsets of genes involved in the acute inflammatory response at 2 h and recovery at 6h. The observed upregulation of clock genes 4 h post-LPS in equine blood supports a role for the molecular clock in this complex process.

#### ***4.5.2 Effects of LPS treatment on core body temperature and melatonin profiles***

As expected, body temperature rose dramatically in LPS treated mares in the hours directly following administration. In addition, the subsequent 24-h mean temperatures were noticeably reduced compared to control mares, possibly signifying a more prolonged disruption of this circadian biomarker. The rise in body temperature at CT22 in LPS-treated mares occurs 4 h before the normal circadian rise in body temperature of control mares, suggesting a phase advancing effect of LPS on the equine circadian system. LPS produces photic-like phase delays of the locomotor activity rhythms of mice when administered at the beginning of the subjective night (Marpegan *et al.* 2005). Further studies of the effects of LPS on the equine circadian system would benefit from extended sampling times, to determine whether an acute systemic inflammation has a phase shifting effect that is sustained over a second 24-h cycle, and administration of the LPS stimulus at different circadian times. Differential circadian responses to endotoxin treatment at different times in the 24-h cycle have been observed in mice (Marpegan *et al.* 2005). There is also evidence that members of the suppressors of cytokine signaling (SOCS) family, potent downregulators of signaling by pro-inflammatory cytokines, are expressed in the SCN with a circadian profile (Sadki *et al.* 2006). Combined, these reports suggest that the circadian system

may vary in its responsiveness to an immune challenge over the 24-h cycle. Melatonin levels appeared unaffected by the inflammatory response and continued to reflect the entraining light/dark period.

#### ***4.5.3 Co-administration of LPS and phenylbutazone (PBZ) blocks clock gene upregulation and the febrile response in vivo***

PBZ is a non-selective cyclooxygenase (COX) inhibitor commonly used as an anti-inflammatory agent in horses. Its inhibitory activity on blood cyclooxygenases, rate-limiting enzymes in the PGE<sub>2</sub> biosynthetic pathway (Ivanov and Romanovsky 2004), has previously been reported (Beretta *et al.* 2005) as has its ability to significantly reduce PGE<sub>2</sub> levels in horses (Morton *et al.* 2005). The observed rise in *Per2* and *Bmal1* expression in equine peripheral blood following LPS administration alone was blocked by co-administration of LPS and PBZ. This result supports the hypothesis that PGE<sub>2</sub> may act as a peripheral clock-resetting agent (Tsuchiya *et al.* 2005). PBZ treatment also blocked the febrile response associated with an endotoxin challenge, further supporting its inhibition of PGE<sub>2</sub>, the principal mediator of fever (Petrovsky and Harrison 1997). In addition, PBZ inhibited the rise in transcript levels of TNF $\alpha$  in circulation. COX inhibitors have previously been shown to inhibit cytokine production by LPS-stimulated human PBMCs (Komatsu *et al.* 1991). This may be explained by the recent finding that COX-1 and COX-2 enzymes can differentially regulate both the upstream nuclear factor (NF)  $\kappa$ -B pathway responsible for cytokine transcription and the downstream enzymatic pathways involved in prostaglandin biosynthesis (Choi *et al.* 2006). Significantly, reports indicate that neurons and glial cells within the SCN are sensitive to inflammatory signals, including TNF $\alpha$  (Bentivoglio *et al.* 2006). Therefore, cytokine-to-brain communication cannot be ruled out as a means of inducing SCN signaling to peripheral tissues.

#### ***4.5.4 Stimulation of PBMCs with LPS, PGE<sub>2</sub> or a heat shock does not directly synchronize clock gene expression***

Cell culture studies were employed to determine whether LPS, PGE<sub>2</sub> or a heat shock directly synchronized clock gene expression in equine PBMCs ex vivo. Expression of TNF $\alpha$  was rapidly induced following treatment of PBMCs with LPS, signifying Toll-like receptor 4 signalling in response to endotoxin (Miyake 2004). The inability of LPS to induce clock gene upregulation strongly suggests that neither LPS nor the release of inflammatory mediators from PBMCs in response to LPS are responsible for direct synchronization of clock gene expression in these cells in vivo. The significant effect of time on *Per2* expression is likely an artifact of the cell culture procedure as a similar effect was observed in untreated cells. As an immediate early gene, *Per2* is more sensitive to changes in the cellular environment than many other genes (Albrecht *et al.* 1997; Shearman *et al.* 1997).

PGE<sub>2</sub> failed to induce circadian oscillation of *Per2* expression in equine PBMCs ex vivo and caused significant repression at higher doses. In contrast, serum shock induced a rapid rise in *Per2* expression 1 h following treatment, similar to our results from a previous study using this method to synchronize equine fibroblasts (Murphy *et al.* 2006). This demonstrates that synchronization of equine PBMCs could be achieved by other means. PGE<sub>2</sub> doses were selected based on a previous report of clock gene synchronization at these concentrations in mouse embryonic fibroblast cells (NIH3T3) (Tsuchiya *et al.* 2005). Tsuchiya *et al.* (2005) clearly identify the EP<sub>1</sub> subtype of PGE<sub>2</sub> receptor as the means by which this molecule acts as an in vivo clock-resetting factor in mice. Their results using NIH3T3 cells led to the assumption that PGE<sub>2</sub> acted directly on cells in peripheral tissues to reset clock gene expression. While our results do not support this assumption, they are consistent with the finding that EP<sub>1</sub> receptors are not detectable in human PBMCs (Strong *et al.* 2001). The 4-h delay in the rise of clock gene expression in response to LPS in vivo further supports an indirect effect of PGE<sub>2</sub> and/or other immune modulators on the molecular clock in equine blood.



The synchronized upregulation of *Per2* and *Bmal1* in blood during acute systemic inflammation is unlikely to be a direct effect of increased body temperature on blood cells as the levels of these clock genes did not change in PBMCs exposed to a heat shock mimicking the in vivo febrile response. These observations indicate that the rise in clock gene expression occurs due to biochemical changes induced indirectly by one or more immune mediators and do not occur as a consequence of cellular metabolic changes induced by a rise in body temperature.

#### ***4.5.5 Potential mechanisms mediating clock gene synchronization in blood during systemic inflammation***

The ability of LPS to shift locomotor activity rhythms in mice and induce c-Fos expression, a marker of neuronal activation in brain structures, in the SCN (Marpegan *et al.* 2005), suggests that immune mediators exert effects at the level of the central pacemaker. While intraperitoneal administration of PGE<sub>2</sub> to mice results in marked phase shifts of clock gene expression rhythms in liver, kidney and heart (Tsuchiya *et al.* 2005), these tissues are known to express high levels of the EP<sub>1</sub> receptor supporting its direct effect on these tissues. However, a direct effect on peripheral cell types has not yet been demonstrated ex vivo. Results from our in vivo experiments support the possibility that PGE<sub>2</sub> mediates a signal from the immune system to the central pacemaker during an acute inflammatory event in the horse. In addition, our results reveal that PGE<sub>2</sub> does not directly synchronize clock gene expression in equine PBMCs. While the possibility that the polymorphonuclear component of blood may be responding to PGE<sub>2</sub> cannot be ruled out, it is reasonable to consider alternative indirect signaling pathways.

One possible pathway is neural liver-to-brain communication. The Kupffer cells (Kc) of the liver constitute the largest population of macrophages in the body and release substantial amounts of PGE<sub>2</sub> following peripheral LPS administration. (Li *et al.* 2006). It is thought that the Kc-generated PGE<sub>2</sub> induces endotoxic fever via its transportation by the bloodstream to the ventromedial preoptic-anterior hypothalamus

(POA, the temperature-regulating center of the brain), or activation of hepatic vagal afferents projecting to the medulla oblongata and eventually connecting to the POA (Blatteis *et al.* 2005). With regard to the latter pathway, it is known that PGE<sub>2</sub> receptors are distributed on vagal afferents (Ek *et al.* 1998). However, a study investigating PGE<sub>2</sub> receptor activation on the isolated ferret vagus nerve failed to demonstrate expression of the EP<sub>1</sub> subtype of receptor (Kan *et al.* 2004) suggesting that this pathway may not be responsible for transmitting an immune signal to the circadian system.

In support of the blood-borne pathway, there is evidence that PGE<sub>2</sub> is released into the brain from cerebral vascular cells during inflammation (Lacroix and Rivest 1998; Quan *et al.* 1998; Yamagata *et al.* 2001). Additionally, radiolabelled PGE<sub>2</sub> was recovered in cerebrospinal fluid from the third cerebral ventricle of rabbits following its peripheral i.v. administration (Davidson *et al.* 2001) demonstrating that peripherally produced PGE<sub>2</sub> crosses the blood-brain-barrier. Significantly, expression of the EP<sub>1</sub> receptor has been documented in many different regions of the adult rat brain (Candelario-Jalil *et al.* 2005) supporting the notion that PGE<sub>2</sub> may signal the circadian system via this route. The 4-h lag period between administration of LPS and upregulation of clock genes in equine peripheral blood also favors a humoral rather than a neural signal. How the inflammatory message finally reaches the SCN and ultimately results in phase resetting of peripheral clocks is as yet unknown.

Peripheral administration of LPS exerts significant autonomic, endocrine and behavioral responses that are controlled by the brain. While evidence suggests that PGE<sub>2</sub> may be a good candidate for a clock-resetting agent during acute systemic inflammation, a host of other inflammatory mediators need to be investigated for their role in regulating the circadian clock during an innate immune response. The transient synchronization of clock gene expression in equine peripheral blood highlights for the first time the possible involvement of the circadian system in restoring homeostasis in this dynamic tissue during an inflammatory challenge.

## CHAPTER 5

### **Clock Gene Upregulation by Equine Polymorphonuclear Neutrophils in Response to the Inflammatory Mediator PGE<sub>2</sub>**

#### **5.1 Summary**

Polymorphonuclear neutrophils provide the first line of defense against lipopolysaccharide (LPS) induced acute systemic inflammation. An emerging body of evidence supports a relationship between the circadian and immune systems during such an innate immune response. *Per2* and *Bmal1* are key molecular components controlling mammalian circadian rhythms at the molecular, cellular and physiological levels. Previously, we have demonstrated synchronized upregulation of these genes in equine whole blood following LPS administration in vivo. Subsequent experiments suggested a role for the febrile mediator, PGE<sub>2</sub>, in mediating this response. However, PGE<sub>2</sub> failed to directly stimulate clock gene expression in equine peripheral blood mononuclear cells (PBMCs). For this reason, we wished to determine whether the polymorphonuclear component of blood might be the source of the observed rise in clock gene expression. In contrast to PBMCs, this study demonstrates that ex vivo cultured equine neutrophils actively respond to PGE<sub>2</sub> by upregulating *Per2* and *Bmal1* expression, whereas stimulation with LPS resulted in upregulation of the cytokine TNF $\alpha$  alone. In addition, we show that LPS induces marked neutrophilia and concomitant monocytopenia in equine peripheral blood in vivo, at the time corresponding to the previously observed clock gene rise. We further report that the peak in the PGE<sub>2</sub> mediated endotoxic fever also occurs simultaneously. Combined, our data suggest that neutrophils are the source of the rise in *Per2* and *Bmal1* expression observed in equine peripheral blood following LPS administration, and that this response is likely mediated by PGE<sub>2</sub>. These results provide the first evidence for a potential role of core circadian clock genes in neutrophil function following innate immune activation.

## 5.2 Introduction

The mammalian circadian system provides a temporal frame necessary to maintain physiological homeostasis by using the varying environmental light/dark cycles to entrain biological processes to a 24-h period. The central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus receives light information and transmits the timing signal to peripheral tissues, synchronizing temporal expression of core clock genes in individual cells (Nagoshi *et al.* 2004; Welsh *et al.* 2004; Yoo *et al.* 2004). Peripheral clocks have been identified in almost all tissues examined to date and share a similar sequential pattern of clock gene expression as the SCN, exemplified by the antiphase oscillation of *Per2* and *Bmal1* mRNAs in heart, lung, liver, eye, kidney and pancreas (Andersson *et al.* 2005; Muhlbauer *et al.* 2004; Oishi *et al.* 1998b). In this way, peripheral tissues anticipate environmental changes and can adapt their specific function to the correct time of day by means of tissue-specific circadian regulation of transcription (Desai *et al.* 2004; Kita *et al.* 2002; Storch *et al.* 2002; Yamamoto *et al.* 2004; Zambon *et al.* 2003).

While the circadian system provides a form of predictive homeostasis by activating physiological mechanisms at the time associated with an expected environmental challenge (Moore-Ede 1986), the innate immune system plays an important role in reactive homeostasis by identifying and eliminating invading pathogens that pose a threat to the internal milieu at any time in the 24-h cycle. Inflammation is one of the first responses of the immune system to infection or irritation, and acute systemic inflammation, as occurs in response to the bacterial endotoxin, lipopolysaccharide (LPS), induces profound autonomic, endocrine and behavioral responses that are primarily controlled by the brain (Watkins *et al.* 1995). Efficient crosstalk between the circadian and immune systems would be of benefit to an organism such that under normal non-inflammatory conditions, the circadian system regulates fluctuations in immune parameters and under acute inflammatory conditions, the reactive immune response stimulates resynchronization of core circadian components, thus in each case maintaining or restoring homeostasis. Recent studies demonstrating circadian regulation of pro-inflammatory cytokines (Petrovsky

and Harrison 1997; Petrovsky *et al.* 1998), circulating levels of different leukocyte populations (Born *et al.* 1997; Kusanagi *et al.* 2004) and natural killer cell function (Arjona and Sarkar 2006), as well as circadian responses to endotoxin treatment in mice (Marpegan *et al.* 2005), support the existence of such a relationship. In addition, we have demonstrated that LPS synchronizes expression of the core clock genes *Bmal1* and *Per2* in equine whole blood in vivo (Murphy *et al.* 2007).

Based on previous results in mice that demonstrate a clock-resetting property of the febrile mediator prostaglandin (PG) E<sub>2</sub> (Tsuchiya *et al.* 2005), we previously hypothesized that this pro-inflammatory compound might be the immune modulator responsible for clock gene synchronization in equine peripheral blood during acute systemic inflammation. This idea was further strengthened by the finding that co-administration of LPS and a non-steroidal anti-inflammatory drug (NSAID), which blocked the endotoxin-induced febrile response and indicated simultaneous inhibition of PGE<sub>2</sub> production (Rotondo *et al.* 1988), also inhibited the rise in clock gene expression (Murphy *et al.* 2007). Subsequent *ex vivo* studies of clock gene expression in cultured equine peripheral blood mononuclear cells (PBMCs) revealed that neither LPS nor PGE<sub>2</sub> directly induced clock gene upregulation in this leukocyte population (Murphy *et al.* 2007). These results suggested that the immune-feedback signal to the circadian machinery in blood cells during an innate immune challenge may be mediated by an indirect pathway, possibly via PGE<sub>2</sub> signaling within the central pacemaker in the brain.

However, an alternative interpretation is that a blood cell population other than PBMCs, such as the polymorphonuclear component of blood, is responsible for the synchronized rise in clock gene expression observed during an inflammatory event. Of these, neutrophils are the predominant cell type accounting for 50-60% of all leukocytes in circulation (Haddy *et al.* 1999; Lassen and Swardson 1995). While it is well known that neutrophils provide the first line of defense against bacterial infections by means of phagocytosis, release of cytoplasmic granule contents and production of reactive oxygen intermediates (ROIs) (Witko-Sarsat *et al.* 2000), their genomic response upon activation was traditionally considered to be somewhat static. There is now increasing evidence that neutrophils have a robust transcriptional

response to LPS (McDonald *et al.* 1997; Nick *et al.* 1999), most notably of cytokines, and microarray data has revealed activation of multiple signal transduction systems not previously associated with LPS exposure (Malcolm *et al.* 2003).

Furthermore, as well as inducing profound transcriptional changes in whole blood, LPS also causes dramatic changes in the relative proportions of blood cell populations following endotoxin challenge in rats (Fannin *et al.* 2005). Significantly, the authors reported marked neutrophilia and lymphopenia in the hours following LPS administration and suggest that changes in levels of specific transcripts are directly related to leukocyte subset changes.

To determine whether polymorphonuclear cells are responsible for the previously observed clock gene synchronization in equine whole blood following endotoxin challenge, we investigated whether *ex vivo* stimulation of equine neutrophils with PGE<sub>2</sub> or LPS results in changes in expression levels of the core circadian clock genes, *Per2* and *Bmal1*. In addition, to determine whether the rise in clock gene expression coincides with a rise in neutrophil levels in circulation, we examined the *in vivo* changes in leukocyte populations in equine blood during an acute systemic inflammation.

## **5.3 Methods**

### **5.3.1 Neutrophil Isolation**

Neutrophils were isolated by a previously described method for rapid isolation of polymorphonuclear leukocytes (De *et al.* 2005) with slight modifications. Peripheral blood from a healthy equine donor was directly drawn into 8 ml Vacutainer cell preparation tubes (CPTs) containing sodium citrate (BD Biosciences, San Jose, CA). The tubes were centrifuged for 25 min at 1700 g at room temperature (RT). The plasma and the PBMCs that were separated above the gel lock were discarded. The upper portion of the gel was washed twice with cold phosphate buffered saline (PBS: 5 ml for each wash). A 3 mL syringe was attached to an 18 gauge 1.5 in. needle and then pierced through the gel to collect the

erythrocyte/neutrophil mixture into the syringe. The needle was removed and the cells were collected into 50 ml centrifuge tubes. The cells were washed with cold  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  - free PBS (Sigma, Saint Louis, Missouri) containing 2.5 % autologous serum by centrifugation for 10 min at 400 g at 4 °C. The erythrocytes were lysed with EL buffer (Qiagen, Valencia, CA) for 10 min at RT with frequent vortexing of the tubes. The cells were washed twice with cold  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  - free PBS by centrifugation for 10 min at 400 g at 4 °C. Cells were then suspended in RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 2.5 % autologous serum, 100 U/mL streptomycin/penicillin and 2 mM L-glutamine (Sigma).

### ***5.3.2 Preliminary investigation of clock gene expression in $\text{PGE}_2$ or LPS treated neutrophils***

Following isolation, cells were counted and checked for viability by trypan blue exclusion on a Vicell<sup>TM</sup>XR Cell Viability Analyser (Beckman Coulter, Fullerton, CA) immediately prior to incubation and again at 4 h post treatment. Neutrophils ( $4 \times 10^6$  cells/mL) were left untreated (control) or treated with LPS (1  $\mu\text{g/mL}$ ) or  $\text{PGE}_2$  (10  $\mu\text{M}$ ) and incubated in suspension for 4 h at 37 °C in a 5 %  $\text{CO}_2$ /air mixture. Following incubation, cells were washed with  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  - free PBS and total RNA was isolated using the RNeasy<sup>®</sup> mini kit (Qiagen) with an additional on-column DNA digestion using the RNase-Free DNase set (Qiagen). Total RNA was quantitatively assessed using the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (Agilent Technologies, Palo Alto, CA).

### ***5.3.3 Neutrophil culture conditions and RNA isolation***

Following a separate isolation, cells were counted and checked for viability by trypan blue exclusion on a Vicell<sup>TM</sup>XR Cell Viability Analyser (Beckman Coulter). Neutrophil purity was determined by flow cytometry. For this, cells were indirectly stained with monoclonal antibodies (mAbs) directed against equine antigens CD172 (clone DH59B, VMRD, Pullman, USA), which stains both neutrophils and

monocytes; CD14 (clone big 10, Biometec, Grefswald, Germany), which is specific for monocytes; or CD5, (courtesy of Dr. D.P. Lunn, University of Colorado) specific for lymphocytes; or with a control mAb of the same isotype (MOPC-21, mouse IgG1, SIGMA). The specificity of the mAbs has been previously demonstrated (Lunn *et al.* 1991; Steinbach *et al.* 2005; Tumas *et al.* 1994). Cells (500,000) were incubated with the mAbs (500 ng, unless specified by the manufacturer) for 30 min at 4°C and washed twice in cold  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  - free PBS at 300 g for 5 min. After 15 min of incubation with 500 ng of FITC-labeled F(ab')<sub>2</sub> fragments of goat anti-mouse Ig (H+L) (Caltag), the cells were washed twice, fixed in 1% paraformaldehyde and analyzed by flow cytometry as described subsequently for whole blood. [Flow cytometry was conducted by Dr. Catherine Méran, University of Kentucky].

Neutrophils ( $4 \times 10^6$  cells/mL) were left untreated or treated with LPS (1 µg/mL) or PGE<sub>2</sub> (100 nM, 1 and 10 µM) and incubated in suspension for 4 h at 37 °C in a 5 % CO<sub>2</sub>/air mixture. Following incubation, cells were washed with  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  - free PBS and total RNA was isolated using the RNeasy<sup>®</sup> mini kit (Qiagen) with an additional on-column DNA digestion using the RNase-Free DNase set (Qiagen). Total RNA was quantitatively assessed using the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (Agilent Technologies).

#### **5.3.4 Real Time RT-PCR**

Equine *Per2*, *Bmal1*, *TNFα*, *β-glucuronidase (GUS)* and *β-Actin* were identified, isolated and Taqman primers and probes designed as previously described (Murphy *et al.* 2007) with the exception of *β-Actin*. The relevant *β-Actin* sequences are: forward primer 5' -gccgtcttcccctccat- 3'; reverse primer 5' -gccacgtatgagtccttctg- 3'; probe 5' -ggcaccagggcgtgatgtgggc- 3'. Both *GUS* and *β-Actin* were first tested for their suitability as endogenous controls in equine neutrophils by determining whether their expression varied significantly across treatments. Gene expression levels are expressed as the number of transcripts per number of molecules of the endogenous control and are reported as a percentage of the peak value.



### **5.3.5 Animals**

Four healthy mares (*Equus caballus*) of mixed light horse breed ranging in age from 3 to 4 years were used in this study. These were the same 4 mares that were used in a previous study demonstrating clock gene upregulation in peripheral blood in response to endotoxin induced acute systemic inflammation (Murphy *et al.* 2007). Two days prior to the experiment mares were housed indoors in individual stalls and allowed ad libitum access to hay and water. Beginning at 7am on the control day, blood samples were collected by jugular venipuncture and core body temperature readings were determined per rectum at 4-h intervals for 12 h. 48 h later, .045 µg/kg LPS was administered i.v. following baseline samples at 7am. This dose was chosen based on previous experiments in our laboratory and previously published equine studies demonstrating the effectiveness of this dose at approximating naturally occurring endotoxemia (King and Gerring 1988; Morris *et al.* 1990; Sprouse *et al.* 1987). Blood samples were collected and body temperature readings recorded 1, 4, 8 and 12 h post LPS treatment. An additional body temperature reading was taken at 2 h post LPS in order to more closely monitor the condition of the animals.

### **5.3.6 Hematology**

Blood samples were collected into EDTA Vacutainer tubes (BD Biosciences) and measured for total and differential leukocyte counts using the Idexx QBC VetAutoread hematology system (Idexx Laboratories, INC., Westbrook, ME) within 30 min of collection. This machine is a quantitative buffy coat analyzer and separates cells using centrifugation followed by measurement of the different heights of each cell layer within a microhaematocrit tube. The buffy coat layer is expanded using a small float, and a fluorescent dye stains the cells to aid differentiation (Pinches 2006). Changes in neutrophil and lymphocyte/monocyte cell populations as well as total WBC (white blood cell) counts are reported as millions of cells/mL.

### ***5.3.7 Whole blood immunostaining and flow cytometry analysis***

The changes in leukocyte populations were assessed by flow cytometry following whole blood staining. 50 µl of blood were incubated for 15 min at room temperature with the same mAbs as for neutrophils. The red blood cells were then lysed with 1 ml of a hypertonic solution (ammonium chloride 1.5 M, sodium bicarbonate 100 mM, disodium EDTA 10 mM), for 10 min. The blood cells were centrifuged for 5 min at 540 g and washed in PBS in the same conditions. Following 15 min of incubation with the FITC-anti-mouse antibody, they were washed twice in PBS at 300 g and fixed in 1 % paraformaldehyde. The cells were kept at 4°C before flow cytometry data acquisition within 24 h. Prior to each acquisition process, a FacsCalibur flow cytometer (BD Biosciences) was calibrated with standard beads (Calibrite 3, BD) using the Facs Comp Software. Data from 50,000 events were recorded for each staining, using the Cell Quest Pro Software (BD Biosciences), with the same settings for every sample. The percentage of leukocyte populations was determined by their surface expression of CD172 (neutrophils and monocytes), CD14 (monocytes) or CD5 (lymphocytes). [Flow cytometry generously conducted by Dr. Catherine Mé rant, University of Kentucky].

### ***5.3.8 Statistics***

One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparisons to a control were used to assess variation in gene expression between treatments in cultured neutrophils. Differences in leukocyte population and body temperatures were determined by two-way (time x treatment) repeated-measures ANOVA followed by Bonferroni post hoc tests. Data were analyzed using Graph Pad Prism Version 4.0 for Windows (Graph Pad Software, San Diego, CA) and are presented as the means  $\pm$  SEM. A value of  $p < .05$  was considered significant.

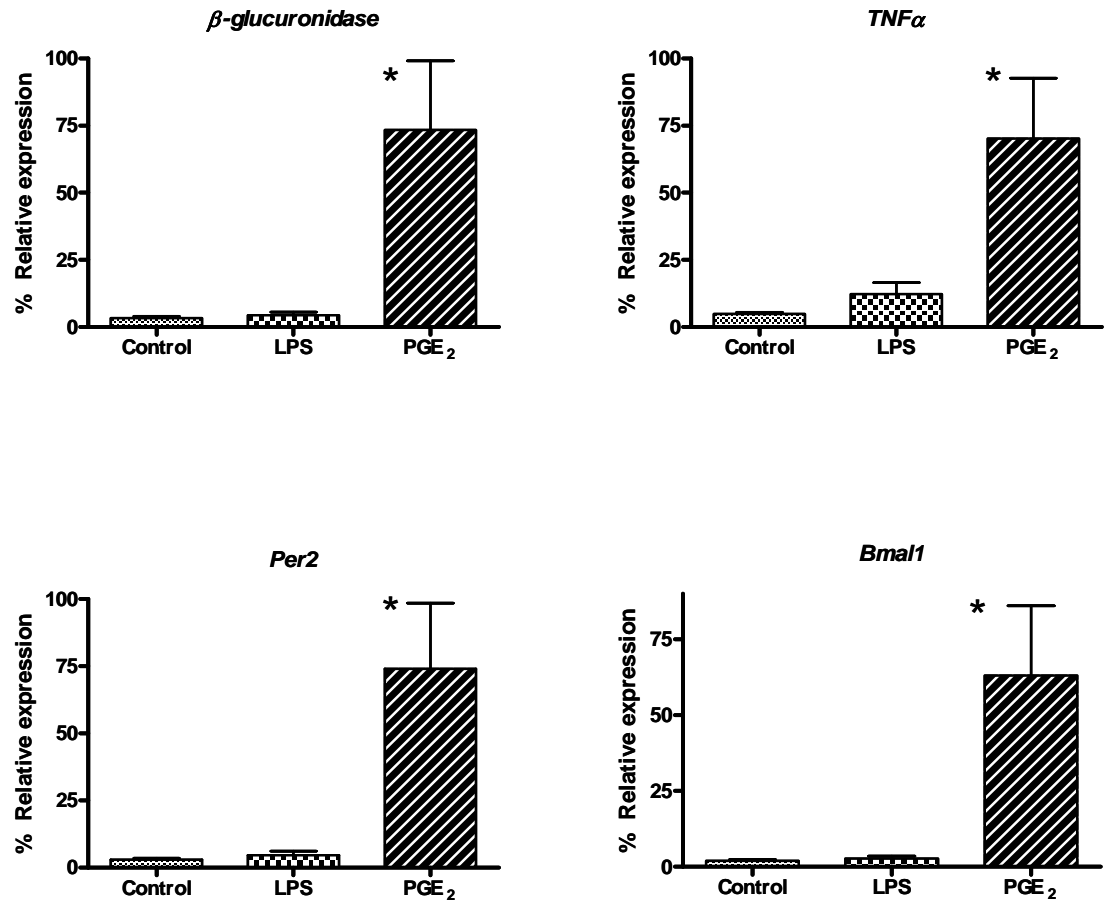
## 5.4 Results

### 5.4.1 Preliminary Results

Prior to incubation, neutrophil viability was determined as 65% and decreased to 55% following 4 h in culture. Unexpectedly, when transcript levels of *GUS* were normalized relative to starting concentrations of Total RNA, to test for suitability as an endogenous control gene, large statistical differences in expression were revealed (Figure 5.1; Table 5.1). *GUS* was found to be an unsuitable control gene given that a 4-fold increase in transcript levels was observed in response to PGE<sub>2</sub> (Table 5.1). This upregulation was considerably amplified once the data was normalized with respect to the different concentrations of RNA in each assay (Figure 5.1), an acceptable alternative form of data normalization when a suitable control gene is not available (Bustin 2000). For this reason, expression levels of *Per2*, *Bmal1* and *TNF $\alpha$*  were also normalized to Total RNA values. One way ANOVA revealed significant upregulation of all genes in response to PGE<sub>2</sub> ( $p < .05$ , respectively; Figure 5.1).

**Table 5.1** Total RNA concentrations and corresponding *GUS* transcript levels as detected by Real Time RT-PCR from triplicate samples of Control, LPS and PGE<sub>2</sub> treated neutrophils.

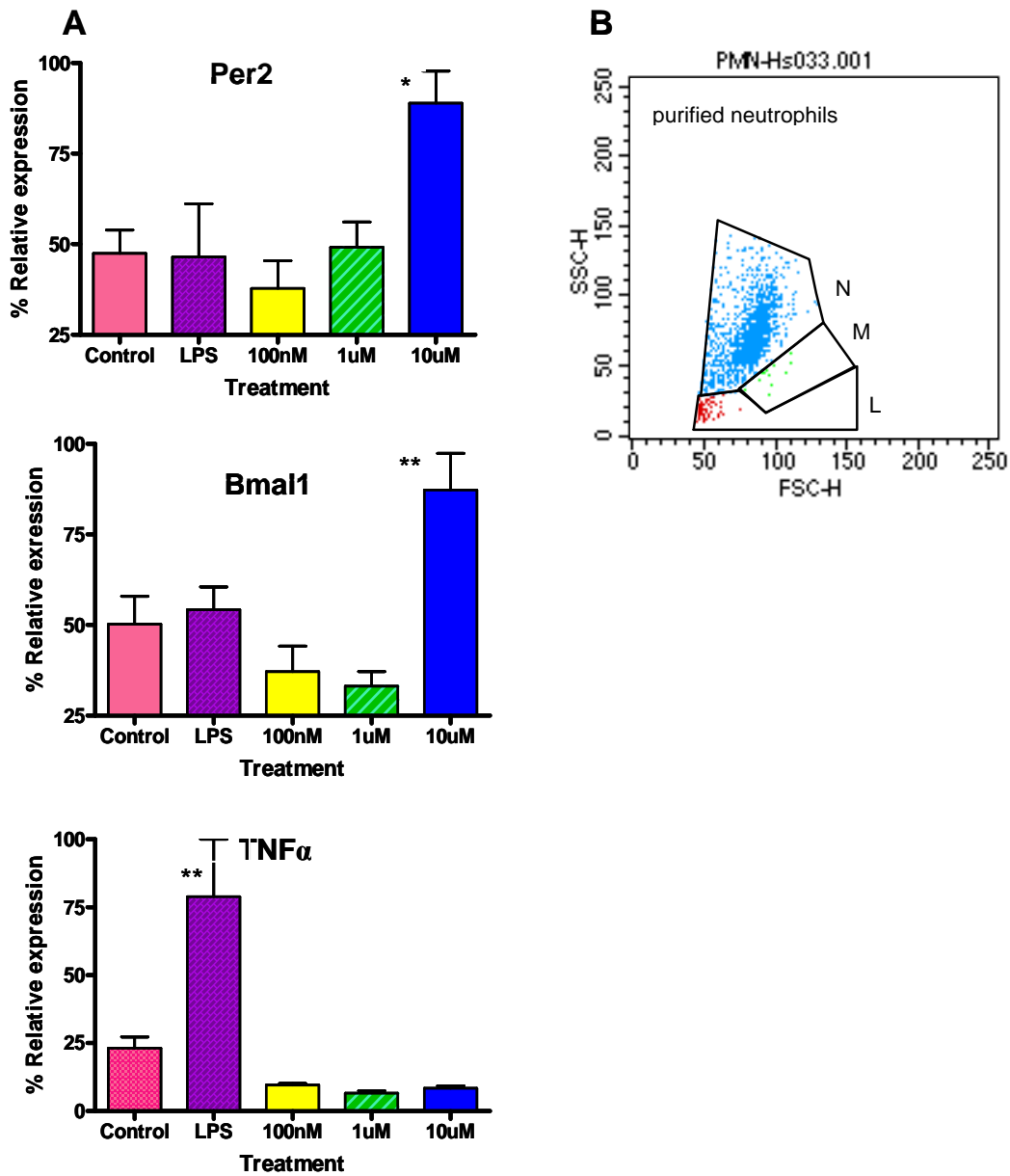
Sample	ng/ $\mu$ l Total RNA	GUS transcripts/5 $\mu$ l RNA
Control 1	29.08	155
Control 2	21.13	200
Control 3	23.3	137
LPS 1	22.24	133
LPS 2	23.37	152
LPS 3	20.68	290
PGE (10 $\mu$ M) 1	4.22	837
PGE (10 $\mu$ M) 2	4.48	905
PGE (10 $\mu$ M) 3	14.88	659



**Figure 5.1** mRNA expression levels of *β-glucuronidase*, *TNFα*, *Per2* and *Bmal1* in equine neutrophils cultured for 4 h in media alone (Control), or supplemented with 1 μg/mL LPS or 10 μM PGE<sub>2</sub>. Gene expression levels are expressed as the number of transcripts relative to starting concentrations (ng/μl) of Total RNA as determined by the Nanodrop<sup>®</sup> ND-1000 Spectrophotometer (Agilent Technologies) and reported as a percentage of the peak value. The data are represented as means ± SEM of three independent experiments. \* denotes significant difference ( $p < .05$ ) from control values.

#### 5.4.2 *Per2*, *Bmal1* and *TNF $\alpha$* expression in neutrophils treated with LPS or PGE<sub>2</sub>

Prior to incubation, neutrophil viability was determined as 88% and decreased to 86% following 4 h in culture. The cultured neutrophil population was determined as greater than 90 % pure by flow cytometry (Figure 5.2B). *Per2* expression in equine neutrophils increased significantly ( $F(13) = 4.62, p < 0.05$ ) following 4 h incubation with 10  $\mu$ M PGE<sub>2</sub>. Expression of this gene was unaffected by treatment of cells with LPS or a lower dose of PGE<sub>2</sub>. Similarly, *Bmal1* expression increased significantly ( $F(13) = 8.59, p < .01$ ) following treatment of neutrophils with 10  $\mu$ M PGE<sub>2</sub>. Again, expression of this clock gene were unaffected by treatment with LPS or a lower dose of PGE<sub>2</sub>. However, LPS treatment of neutrophils resulted in significant ( $F(13) = 18.34, p < .001$ ) upregulation of the pro-inflammatory cytokine *TNF $\alpha$* . Changes in gene expression for all three genes are shown in Figure 5.2A.



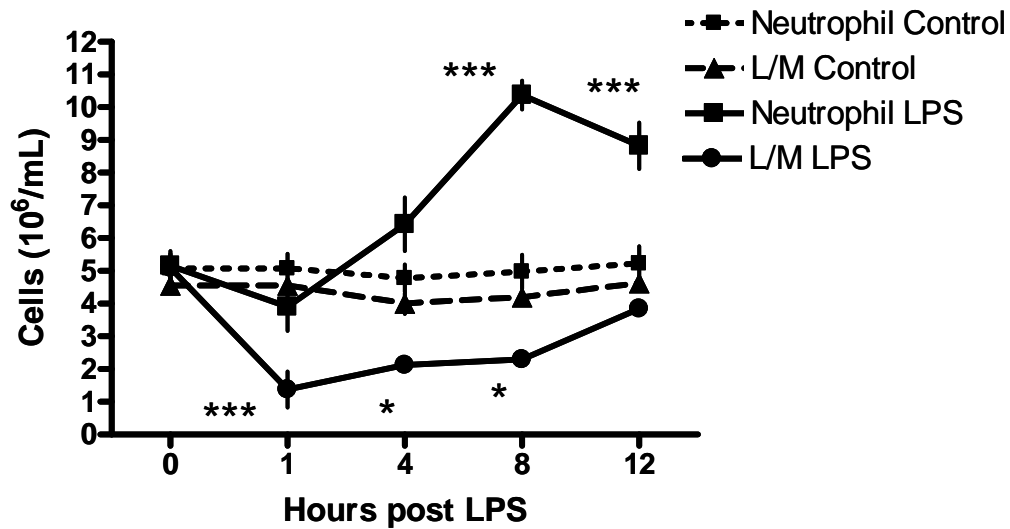
**Figure 5.2** (A) mRNA expression levels of *Per2*, *Bmal1* and *TNFα* in equine neutrophils cultured for 4 h in media alone, or supplemented with 1 µg/mL LPS or 100 nM, 1 µM and 10 µM PGE<sub>2</sub>. Gene expression levels are expressed as the number of transcripts per number of molecules of the internal control gene β-actin and are reported as a percentage of the peak value. The data are represented as means ± SEM

of three independent experiments. \*, \*\* denote significant difference ( $p < .05$ ,  $p < .01$ , respectively) from control values. (B) Assessment of neutrophil purity by fluorescent immunostaining. Cells were stained with anti-equine CD172, CD14 and CD5 mAbs and analyzed by flow cytometry. Back-gating with these stainings indicates that the isolated cells were neutrophils (N), in contrast to whole blood cells (see Figure 5.5). M: monocytes, L: lymphocytes.

#### ***5.4.3 Leukocyte population changes during endotoxin induced acute systemic inflammation in equine blood***

Acute systemic inflammation resulted in marked neutrophilia and lymphocytopenia in equine blood (Figure 5.3; Table 5.2). Absolute neutrophil counts, as determined by hematology analysis, initially decreased by 23 % from control levels at 1 h post LPS treatment and then rapidly increased to 35 %, 108% and 69 % greater than control levels at 4 h, 8 h and 12 h, respectively. Combined lymphocyte/monocyte (L/M) levels decreased by 70 % at 1 h post LPS treatment and remained at 47 %, 45 % and 17 % below control levels at 4 h, 8 h and 12 h, respectively. Overall, total white blood cell (WBC) counts dropped by 50% at 1 h post treatment, returned to control levels at 4 h and increased to 38 % and 29 % greater than controls at 8 h and 12 h, respectively (Figure 5.4). There were no significant differences between neutrophil and L/M counts at any time point on the control day. In contrast, two-way repeated measures ANOVA revealed a significant time x treatment interaction ( $F(12,4) = 20.11$ ,  $p < .0001$ ) and significant effect of time ( $F(4) = 22.33$ ,  $p < .0001$ ) and treatment ( $F(3) = 25.85$ ,  $p < .0001$ ) on neutrophil and L/M levels following LPS. Post hoc tests revealed significant ( $p < .001$ ) differences between groups at each time point following the baseline values at 0 h. These results were supported by almost identical results using the flow cytometry method of cell count determination. Slight variations in absolute cell counts between methods were not reflected in any differences in statistical significance. As shown in Figure 5.5 (whole blood dot plots), the neutrophil population was reduced 1 h after LPS injection, but expanded 4 h and 8 h post LPS. In contrast, the mononuclear cell pool

(lymphocytes and monocytes) was still reduced at 4 h, 8 h and 12 h. Absolute neutrophil, lymphocyte and monocyte counts calculated from flow cytometry percentages confirmed that LPS induced significant neutrophilia (data not shown).



**Figure 5.3** Peripheral blood leukocyte changes over time in control and LPS treated horses as determined by hematology analysis. The data are expressed as millions of cells per mL of blood and are represented as means  $\pm$  SEM ( $n = 4$ ) per time point. L/M: Combined lymphocyte and monocyte counts. \*, \*\*\* denote significant difference ( $p < .05$ ,  $p < .001$ , respectively) from control values at corresponding time points.



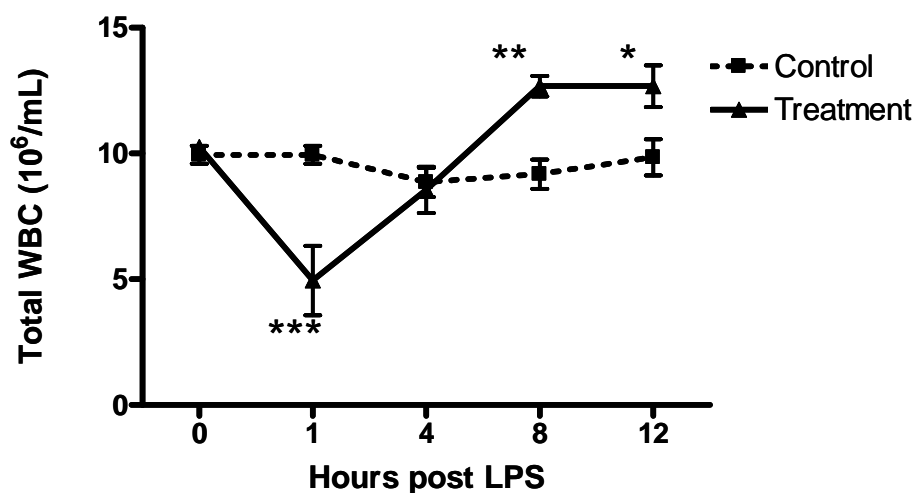
**Table 5.2** Mean absolute values of leukocyte differentials

	Control <sup>1</sup> 1 h	LPS <sup>1</sup> 1 h	Control <sup>1</sup> 4 h	LPS <sup>1</sup> 4 h	Control <sup>1</sup> 8 h	LPS <sup>1</sup> 8 h	Control <sup>1</sup> 12 h	LPS <sup>1</sup> 12 h
Total WBC <sup>2</sup>	9.9±.4	4.9±1.3	8.8±.6	8.5±.9	9.2±.6	12.7±.4	9.8±.7	12.7±.8
Neutrophils	5.1±.4 (55.5)	3.9±.7 (79.6)	4.8±.4 (54.5)	6.4±.8 (75.3)	5±.5 (54.4)	10.4±.4 (81.9)	5.2±.5 (53)	8.8±.7 (69.3)
L/M <sup>3</sup>	4.5±.1 (45.5)	1.4±.5 (28)	4±.3 (45.5)	2.1±.1 (24.7)	4±.1 (43.5)	2.3±.2 (18)	4.6±.2 (46.9)	3.8±.1 (29.9)
Number	4	4	4	4	4	4	4	4

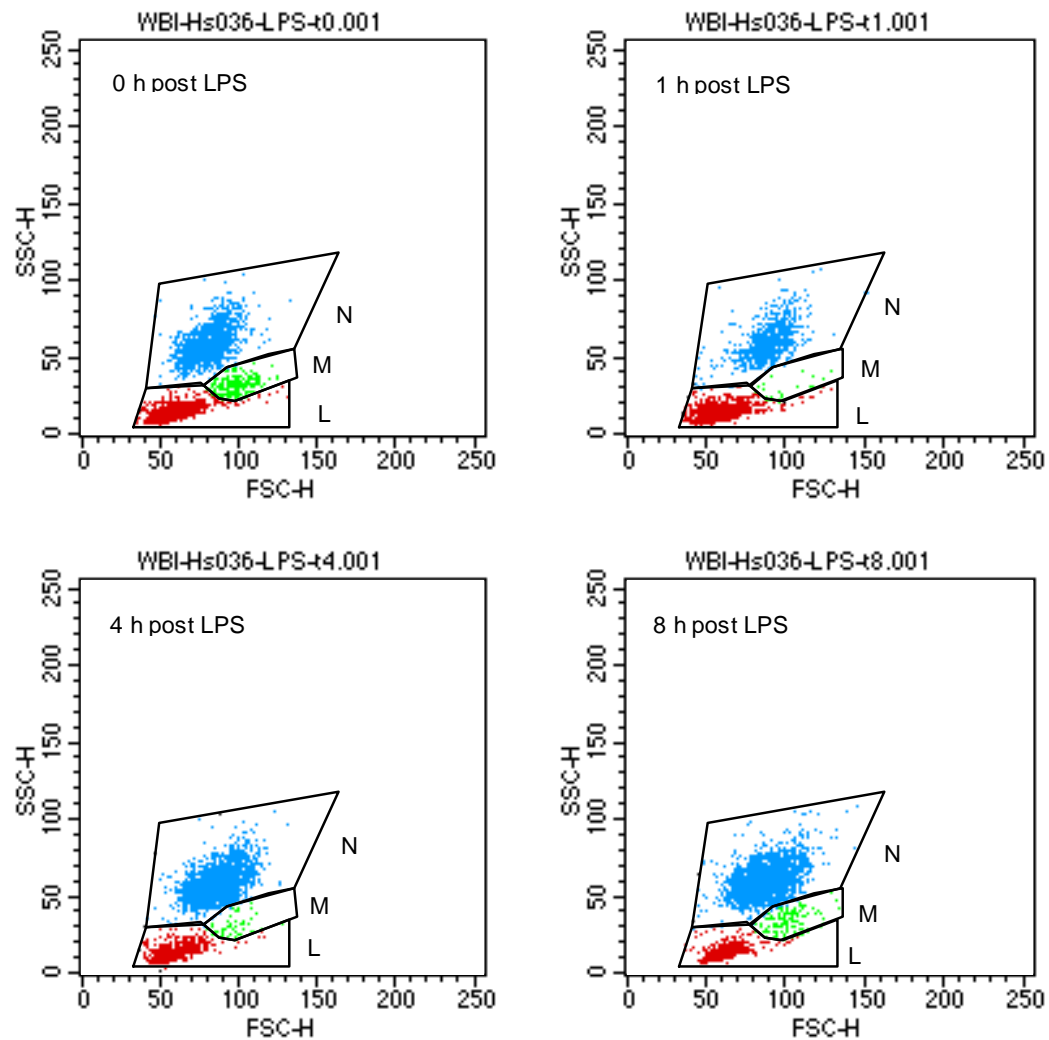
<sup>1</sup> All values, except number, are means ± SEM. Numbers in parentheses are percentages of total white blood cell (WBC) count

<sup>2</sup> White blood cells

<sup>3</sup> Lymphocytes and monocytes



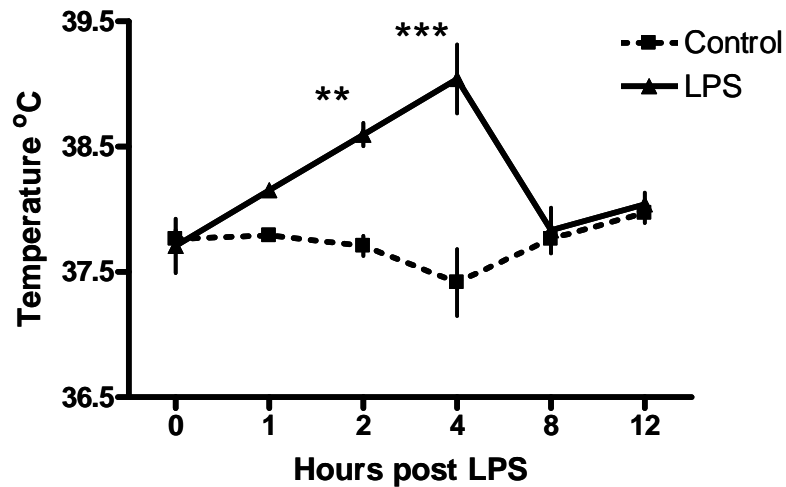
**Figure 5.4** Peripheral blood total white blood cell (WBC) count changes in control and LPS treated horses as determined by hematology analysis. The data are expressed as millions of cells per mL of blood and are represented as means ± SEM (n= 4) per time point. \*, \*\*, \*\*\* denote significant difference ( $p < .05$ ,  $p < .01$ ,  $p < .001$ , respectively) from control values.



**Figure 5.5** Flow cytometry analysis of the changes in the leukocyte composition of equine whole blood after administration of LPS. Whole blood was stained with anti-horse CD172, CD14 and CD5 mAbs. Cells were gated according to their expression of CD172 (neutrophils, monocytes), CD14 (monocytes) or CD5 (lymphocytes). The positivity of the labeling was determined by comparison with the staining given by an irrelevant mouse IgG1. N: neutrophils, M: monocytes, L: lymphocytes.

#### 5.4.4 Core Body Temperature changes during endotoxin induced acute systemic inflammation

Changes in body temperature over a 12-h period on the control and LPS treatment days are shown in Figure 5.6. There was a significant time x treatment interaction ( $F(5,1) = 10.42, p < .0001$ ) and a significant effect of time ( $F(5) = 3.65, p < .05$ ) and treatment ( $F(1) = 19.6, p < .001$ ). LPS treatment resulted in the expected febrile episode beginning 1 h post treatment and peaking at 4 h. Post hoc tests revealed significant differences between groups at 2h and 4 h ( $p < .01$  and  $p < .001$ , respectively).



**Figure 5.6** Changes in core body temperatures over a 12-h period in control and LPS treated horses. The data are represented as means  $\pm$  SEM ( $n = 4$ ) per time point. \*\*, \*\*\* denote significant difference ( $p < .01, p < .001$ , respectively) from control values.

## 5.5 Discussion

### 5.5.1 $PGE_2$ stimulates upregulation of *Per2* and *Bmal1* in equine neutrophils

In contrast to other species, there is no evidence of synchronized clock gene expression in equine peripheral blood from normal, healthy animals (Murphy *et al.* 2006). However, induction of an acute inflammatory response by administration of

LPS stimulates synchronous elevated transcription of *Per2* and *Bmal1* that reaches maximal levels 4 h post-treatment (Murphy *et al.* 2007). Inhibition of the rise in *Per2* and *Bmal1* expression by co-administration of the NSAID, phenylbutazone, a known suppressor of cyclooxygenase-2 synthesis in equine tissues (Beretta *et al.* 2005), suggested that the effects of LPS on clock gene transcription in equine blood were mediated through PGE<sub>2</sub>, rather than through Toll-like receptor-4 complexes. This conclusion is not without precedent since PGE<sub>2</sub> has been demonstrated to induce or reset circadian rhythms in clock gene expression in mouse fibroblast (NIH3T3) cells and mouse peripheral tissues (Tsuchiya *et al.* 2005). The results presented here support the hypothesis that the rise in *Per2* and *Bmal1* expression observed in equine peripheral blood following LPS administration is mediated by PGE<sub>2</sub>. Addition of this pro-inflammatory compound to enriched populations of equine neutrophils induced transcription of *Per2* and *Bmal1*, whereas LPS did not. While this effect was only observed for the higher PGE<sub>2</sub> concentration, this likely reflects in vitro conditions that do not adequately mimic an in vivo inflammatory response. The accelerated rate of neutrophil apoptosis associated with inflammatory conditions in vivo, facilitated by direct interaction with pathogen and other cells involved in the inflammatory response (Brown and Savill 1999; Kobayashi *et al.* 2003), may have a synergistic effect on clock gene upregulation at lower physiological PGE<sub>2</sub> concentrations. This would explain the robust rise in clock gene expression previously observed in response to LPS in vivo (Murphy *et al.* 2007). In fact, the results from our preliminary experiment demonstrated significantly greater fold increases in clock gene expression in response to PGE<sub>2</sub> when associated with reduced neutrophil viability prior to treatment.

Furthermore, upregulation in *Per2* and *Bmal1* mRNA synthesis in PGE<sub>2</sub> treated neutrophils occurred simultaneously, similar to our previous observations in whole blood (Murphy *et al.* 2007). This is in contrast to the normal temporal profiles observed in the SCN and most peripheral tissues (including equine adipose tissue), where an antiphase expression of these genes occurs (Andersson *et al.* 2005; Muhlbauer *et al.* 2004; Oishi *et al.* 1998b). Although LPS had no measurable effect on clock gene expression in enriched equine neutrophil cultures, it did cause a

significant increase in *TNF $\alpha$*  mRNA levels. This supports recent findings that these short-lived cells are transcriptionally active (Malcolm *et al.* 2003) and contribute more to innate immune responses than just the release of pre-made inflammatory mediators (McDonald *et al.* 1997; Nick *et al.* 1999).

#### ***5.5.2. Core body temperature and leukocyte population changes in response to LPS in vivo***

The LPS induced rise in body temperature reached its peak at 4 h post-treatment and therefore corresponded with maximal *Per2* and *Bmal1* expression levels observed in equine blood in our previous experiments using the same animals (Murphy *et al.* 2007). PGE<sub>2</sub> is required for the induction and maintenance of febrile responses (Blatteis 2006; Ivanov and Romanovsky 2004; Li *et al.* 2006; Ushikubi *et al.* 1998). As circulating concentrations of this molecule are closely correlated with increases and decreases in core body temperature (Blatteis 2006; Davidson *et al.* 2001), it is expected that concentrations of PGE<sub>2</sub> in blood would be at their highest 4 h post-LPS treatment.

In addition to inducing fever, LPS treatment had profound effects on the numbers and relative proportions of different equine blood cell populations. These changes in leukocyte population kinetics were found to be almost identical to those observed following LPS administration in humans (Richardson *et al.* 1989). In both species, LPS caused a pronounced neutrophilia with concomitant decreases in PBMCs. Although the neutrophilia did not peak until 8 h, at 4 h post-LPS treatment, when circulating levels of PGE<sub>2</sub> are likely to be at their highest numbers, neutrophils were 35 % higher and PBMCs 53 % lower compared to control levels. Therefore, observations from this and our previous study demonstrating the inhibitory effects of phenylbutazone (Murphy *et al.* 2007), provide strong correlative evidence that following an acute inflammatory stimulus, PGE<sub>2</sub> acts directly on neutrophils to trigger the synchronous, enhanced expression of *Per2* and *Bmal1*. Furthermore, detection of this effect is increased by LPS induced expansion of the circulating neutrophil population.

These results provide the first evidence for a potential role of core circadian clock genes in neutrophil function following activation of innate immune responses. This is not the first study to identify a role for clock genes in immune cell function. Recent evidence also highlighted an essential role for *Per2* in gamma interferon release from natural killer (NK) cells during LPS induced endotoxic shock in mice (Liu *et al.* 2006). While the role of clock gene upregulation in neutrophils remains to be further characterized, PGE<sub>2</sub> is known to regulate a range of functions in human neutrophils including chemotaxis (Armstrong 1995), superoxide anion generation (Talpain *et al.* 1995; Wheeldon and Vardey 1993), apoptosis (Liu *et al.* 2000; Rossi *et al.* 1995) and differential cytokine production (Yamane *et al.* 2000). It is now conceivable that clock gene expression may be involved in some or all of these processes. In this regard it is important to note that the CLOCK protein, which is the heterodimerization partner of BMAL1, has been shown to function as a histone acetyltransferase with chromatin remodeling activity that is significantly enhanced when bound to BMAL1 (Doi *et al.* 2006). Therefore, PGE<sub>2</sub> mediated upregulation of *Bmal1* expression likely induces epigenetic changes in neutrophil chromosomal DNA resulting in altered patterns of gene transcription. This hypothetical role for PGE<sub>2</sub> is in keeping with the recently reported ability of neutrophils to transcriptionally activate a number of signal transduction systems in response to LPS (Malcolm *et al.* 2003). However, their ability to upregulate the core clock genes *Per2* and *Bmal1* was hitherto unidentified in Malcolm *et al.*'s model highlighting genes involved in LPS induced neutrophil activation. This serves to underline the difficulty in interpreting in vitro experiments intending to ascertain the effects of LPS on specific cell types. Under in vivo conditions, neutrophils are exposed to a multitude of pro- and anti-inflammatory stimuli, including increased levels of PGE<sub>2</sub>, which can have compounding and synergistic effects on transcriptional changes.

### ***5.5.3 Significance of findings from preliminary neutrophil challenge***

The results of the initial experiment that demonstrated a significantly greater transcriptional response of neutrophils to PGE<sub>2</sub> than the subsequent experiment merits further discussion. The only perceptible difference between the two neutrophil

challenges was the percent viability of the cells prior to treatment and incubation. This was likely due to lack of prior experience with the neutrophil isolation technique resulting in greater than optimal time duration between blood collection and incubation of isolated neutrophils. In addition, less careful handling of the cells during the preliminary isolation also potentially contributed to increased cell death. Detection of cell viability by Trypan Blue exclusion is limited to detecting cells whose membranes have become permeable in the final stages of cell death, resulting from either apoptotic or necrotic processes. The drawback of this technique is that the earlier stages of apoptosis cannot be detected. Based on the low percent viability of isolated cells during the preliminary challenge, it is likely that an even greater percent were actively undergoing the earlier, undetectable stages of apoptosis. Ironically, the conditions of this experiment, while not considered ideal by cell culture practices, probably more accurately reflect in vivo conditions during inflammation. Neutrophils rapidly undergo apoptosis once their defensive roles to destroy and digest invading microorganisms at sites of inflammation have been accomplished, in order that their potentially histotoxic contents are rapidly cleared from circulation (Nathan 2002). However, the transcriptional regulation of this process during bacterial infection is only beginning to be elucidated (Kobayashi *et al.* 2003; Walker *et al.* 2005). Our results underline how the status of the cell in the presence of the febrile mediator, PGE<sub>2</sub>, may affect the level of transcriptional activity for a number of important genes.

Activity of the enzyme  $\beta$ -glucuronidase (*GUS*) is known to increase in rat plasma in response to i.v. injected LPS (Shimoi *et al.* 2000; Shimoi *et al.* 2001) and its role in deglucuronidation (hydrolysis of steroid/estrogen hormone glucuronides) (Zhu *et al.* 1996) during inflammation is established (Shimoi and Nakayama 2005). However, increased activity of this enzyme was in all cases attributed to granulocyte release of lysosomal contents. Only in one case did the authors suggest that the expression level of *GUS* might be higher in inflammatory cells, such as neutrophils, than in normal tissue cells (Shimoi and Nakayama 2005). The results from the preliminary experiment provide possibly the first evidence that apoptotic neutrophils actively transcribe significant amounts of *GUS* in response to the inflammatory mediator PGE<sub>2</sub>. While to some extent speculative, these suggestions are plausible and

explain the high level of *GUS* activity observed in inflammatory tissue and the fact that this gene was not identified among upregulated genes in neutrophil response to LPS alone (Malcolm *et al.* 2003).

Furthermore, while it is well known that LPS stimulates the production of cytokines from neutrophils (Cassatella 1995), the results of the neutrophil challenges presented here may suggest that PGE<sub>2</sub> differentially stimulates or suppresses *TNFα* expression depending on the status of the cells. Overall, it is clear that further insights will be gained in elucidating transcriptional signaling mechanisms involved in the resolution of inflammation by exogenously inducing neutrophil apoptosis and challenging the cells with individual inflammatory mediators.

The results of these experiments both bring to light a new relationship between the circadian and immune systems in response to a homeostatic challenge and highlight the importance of further investigation of neutrophil function in response to an important inflammatory mediator.



## CHAPTER 6

### Investigation of the effects of Jet Lag on the Equine Circadian System

#### 6.1 Summary

Rapid displacement across multiple time zones results in a conflict between the new cycle of light and dark and the previously entrained program of the internal circadian clock, a phenomenon known as jet lag. In humans, jet lag is often characterized by malaise, appetite loss, fatigue, disturbed sleep and performance deficit, the consequences of which are of particular concern to athletes hoping to perform optimally at an international destination. As a species renowned for its capacity for athletic performance, the consequences of jet lag are also relevant for the horse. However, the duration and severity of jet lag related circadian disruption is presently unknown in this species. We investigated the rates of re-entrainment of serum melatonin and core body temperature rhythms following an abrupt 6-h phase advance of the LD cycle in the horse. Six healthy, 2 yr old mares entrained to a 12 h light/ 12 h dark (LD 12:12) natural photoperiod were housed in a light-proofed barn under a lighting schedule that mimicked the external LD cycle. Following baseline sampling on Day 0, an advance shift of the LD cycle was accomplished by ending the subsequent dark period 6 h early. Blood sampling for serum melatonin analysis and core body temperature readings were taken at 3-h intervals for 24 h on alternate days for 11 days. Disturbances to the subsequent melatonin and body temperature 24-h rhythms were assessed using repeated measures ANOVA and analysis of Cosine curve fitting parameters.

We demonstrate that the equine melatonin rhythm re-entrains rapidly to a 6-h phase advance of an LD12:12 photocycle. The phase shift was fully complete on the first day of the new schedule and rhythm phase and waveform were stable thereafter. In comparison, the advance in the body temperature rhythm was achieved by the third day, however body temperature rhythm waveform, especially its mesor, was altered for many days following the LD shift. Aside from the temperature rhythm

disruption, rapid resynchronization of the melatonin rhythm suggests that the central circadian pacemaker of the horse may possess a particularly robust entrainment response. The consequences for athletic performance remain unknown.

[The material in this chapter has been published in *The Journal of Circadian Rhythms* 2007, 21: 467-476]

## 6.2 Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the location of the master mammalian pacemaker that controls circadian rhythms of diverse physiological and behavioral phenomena, including rest-activity cycles, hormone secretion and body temperature. The circadian system provides endogenous timekeeping mechanisms that allow organisms to optimize survival by adaptively anticipating periods of activity and entraining physiological function to the solar day (Buijs *et al.* 2003). Accordingly, light is the primary time cue serving to synchronize circadian rhythms to the 24-hour period of the earth's rotation. Circadian entrainment mechanisms also ensure that behavioral, endocrine and other 24-hour rhythms of the body are phased or timed adaptively with respect to the environment. The SCN receives photic information via the retino-hypothalamic tract and transmits timing signals to synchronize peripheral clocks located throughout the organism (Reppert and Weaver 2002).

In humans, disruption of the circadian timing system occurs in response to rotational shift work and transmeridian travel. Rapid displacement across multiple time zones results in a mismatch between the previously entrained program of the internal circadian clock and the new cycle of light and dark (LD), a phenomenon known as jet lag (Nagano *et al.* 2003; Winget *et al.* 1984). The most common symptoms of human jet lag, including malaise, appetite loss, fatigue and disturbed sleep, last until the circadian clock system adjusts to the new environmental conditions, re-establishing preferred phase relations among different rhythms and between these rhythms and the external environment (Gander *et al.* 1985; Satoh *et al.* 2006). Circadian desynchronization is of particular concern to athletes hoping to perform optimally at an international destination. Significant decreases in reaction times, cardiorespiratory functions and muscle strength have been reported following transmeridian travel in humans (Lemmer *et al.* 2002; Loat and Rhodes 1989; Manfredini *et al.* 1998; Reilly *et al.* 2005) with prolonged disturbance associated with easterly travel (Aschoff *et al.* 1975). The consequences of jet lag are equally relevant for the equine athlete with the high frequency of air travel for international

equestrian competition. However, there are no previous studies examining re-entrainment of the equine circadian system following a phase shift of the LD cycle, and the duration and severity of jet lag related circadian disruption is presently unknown. The precise cellular, organismal, and behavioural mechanisms underlying jet-lag induced reductions in athletic performance, human or equine, remain to be elucidated. Nonetheless, a reasonable first step in understanding the phenomenon may be to characterize the severity and longevity of measurable perturbations in normal circadian organization as can be observed by studying and quantifying the parameters of two or more well characterized circadian rhythms in relation to each other and to the LD cycles employed.

Because it is not possible to directly monitor the functional timing of the endogenous circadian clock, marker rhythms that measure clock output are commonly used to assess circadian phase position. Two common, useful and physiologically important markers of circadian phase have been the circadian rhythms of blood melatonin and core body temperature. The 24-h melatonin profile provides a robust marker of circadian phase in humans (Lewy and Sack 1989; Rosenthal 1991) and has been used to provide reliable estimates of circadian adaptation to phase shifts (Akerstedt *et al.* 1979; Boivin and James 2002; Van Cauter *et al.* 1998). Similarly, body temperature is a commonly assessed marker of circadian phase (Benloucif *et al.* 2005; Klerman *et al.* 2002; Refinetti and Menaker 1992) and has been used to determine rates of re-entrainment in humans (Boivin and James 2002) and rodents (Goel and Lee 1996). Both melatonin and body temperature have previously been shown to exhibit robust 24-h rhythms in the horse (Piccione *et al.* 2005) and an investigation under constant light (LL) confirmed that the various parameters of the equine body temperature rhythm are similar to those of several species of rodents (Piccione *et al.* 2002).

The primary aim of this study was to evaluate the rates of re-entrainment of the equine melatonin and body temperature rhythms following an abrupt 6-h phase advance of the LD cycle, mimicking an eastward transmeridian journey across 6 time zones. We demonstrate that these two markers of circadian phase resynchronize significantly more rapidly in horses than in humans or rodents in response to a similar

phase shift, suggesting a more rapid and robust entrainment response of the central pacemaker in the horse.

## **6.3 Methods**

### **6.3.1 Animals**

Six healthy 2 year old mares (*Equus caballus*) of mixed light horse breed were used in this study. Animals were maintained outdoors under conditions of natural photoperiod prior to the experiment, which was conducted at the time of year (mid-September) corresponding to an approximate 12 h light/12 h dark (LD12:12) natural photoperiod (sunrise at 7:30 AM, sunset at 7.30 PM; longitude 84.5°W, latitude 38.1°N). Five days prior to the experiment, mares were housed in individual stalls in a light-proofed barn under a lighting schedule that mimicked the external photoperiod but with abrupt L/D and D/L transitions (LD 12:12). During the hours of light, stalls were lit by two 200 W light bulbs that produced an average light intensity of 350 lux at eye level. Access to water was *ad libitum* and feed was provided 4 times a day to prevent a conspicuous 24-h temporal cue (Piccione *et al.* 2002). The ambient internal barn temperature varied from 19-21° C during the experimental period. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC).

### **6.3.2 Experimental protocol**

The day before initiation of sample collection, mares were fitted with indwelling jugular catheters. Beginning at 7:30 AM (ZT 0) on Day 0, and ending ~24-h later (ZT 0) blood samples were collected and rectal core body temperature readings were taken by digital thermometer at 3-h intervals (9 samples). Blood samples were allowed to clot and kept overnight at 4°C. The next day, samples were centrifuged and the serum harvested and stored at -20°C until assayed for melatonin. Throughout the experiment, sampling during the hours of darkness was conducted

with the aid of a dim red light from handheld flashlights. Mares were well accustomed to the sampling procedure and were only minimally disturbed by the presence of the sample collector. At each time point, samples were collected from mares in the same order with approximately 3 min intervals between animals. After experiencing one additional 12 h photoperiod at the usual time, an advance shift of the LD cycle was accomplished by ending the dark period 6 h early, resulting in lights on from 1:30 AM to 1:30 PM (ZT 18 became new ZT 0) starting on experiment Day 1 and continuing through Day 11. Beginning at lights on (1:30 AM/new ZT 0), blood sampling and body temperature readings were again taken at 3-h intervals for 24 h (ZT 0 to ZT 0) on alternate days for 11 days. The mares were consistently sampled in the same order at each sampling time with approximately 3 min intervals between animal. Sampling at ZT 0 was conducted immediately following lights-on and at ZT 12, immediately following lights-off.

### **6.3.3 Melatonin Radioimmunoassay (RIA)**

Melatonin was measured by a commercial RIA kit (Alpco, Windham, NH) as described previously (Fitzgerald *et al.* 2000). Briefly, a 1 ml serum aliquot was extracted according to the directions of the manufacturer and reconstituted in a buffer solution provided. Aliquots of the extracted samples were assayed in duplicate. Intra-assay coefficients of variation for low and high melatonin concentration pools were 14% and 5% respectively. The limits of detection of the assay averaged 0.5 pg/ml.

### **6.3.4 Data Analysis**

Melatonin and body temperature time series data were analysed using a computer program based on the least squares cosine fit method of Nelson *et al.* (1979) (Nelson *et al.* 1979). For each mare and each experimental day (9 points, 24 h), this cosinor method gave estimates of 3 rhythm parameters: acrophase (time of peak value of the fitted cosine function), amplitude (half the difference between the minimum

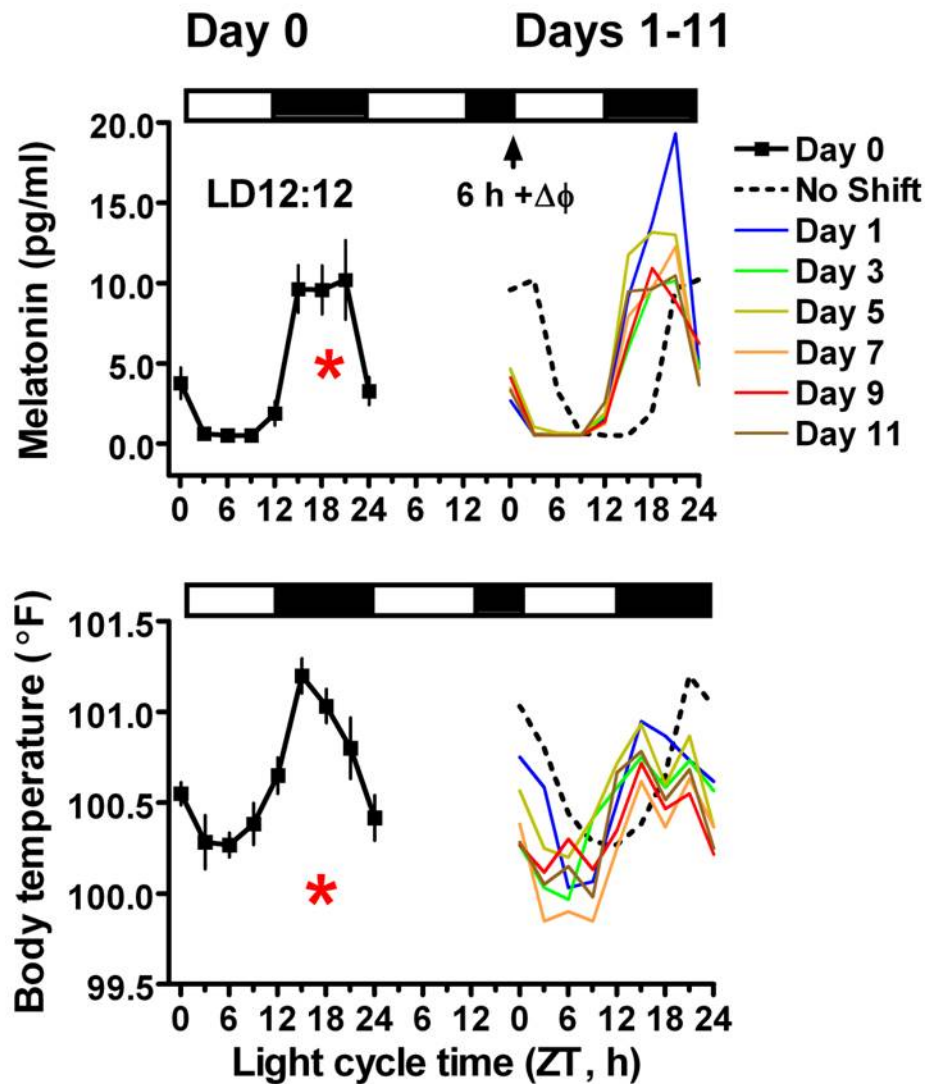
and maximum of the fitted cosine function) and mesor (middle value of the fitted cosine curve representing the rhythm adjusted mean). A potential advantage of the cosinor method is that acrophase is calculated from the entire 24-h time series and represents the mean vector of the circular (24-h) distribution. Also, we chose this method because with 3-h sampling we did not have the temporal resolution to track changes in melatonin or body temperature rhythm phase adjustments using such markers as the onset (evening rise) of melatonin or the 24 h nadir in body temperature. One-way repeated measures ANOVA was used to assess significant changes in mean melatonin and body temperature values and changes in cosinor parameters over time on each individual day (pre- and post-shift). Two-way repeated measures ANOVA (Day X Time) was used to assess differences in melatonin and body temperature rhythms between Day 0 and Days 1-11 at the corresponding ZT times post-shift. Bonferroni post hoc tests were used to evaluate differences between time points where appropriate. Data was analyzed using Graph Pad Prism Version 4.0 for Windows (Graph Pad Software, San Diego, CA,) and are presented as the means  $\pm$  SEM. A value of  $p < 0.05$  was considered significant.

## **6.4 Results**

### ***6.4.1 Baseline Body Temperature and Melatonin Rhythm Assessment***

Individual cosine fits for both melatonin and body temperature profiles were significant for all mares on baseline Day 0 (Figure 6.1). The acrophase of the melatonin rhythm occurred at 1:35 AM (ZT 17.5, Figure 6.3A; Table 6.1), almost exactly midway through the dark period, while the baseline body temperature acrophase occurred at 12:28 AM, (ZT 16.5) one hour and twenty minutes prior to the melatonin acrophase (Figure 6.3A; Table 1). The latter is equivalent to a cosine nadir at ZT 4.5 (12:28 PM). One-way repeated measures ANOVA ( $n = 6$ ) revealed a significant variation in mean melatonin and body temperature values over time ( $p < .0001$ , respectively). melatonin values were low at ZT 0 and dropped to undetectable levels ( $< .5$  pg/mL) at ZT 3, ZT 6 and ZT 9. Following a consistent but slight non

significant rise at ZT 12, post hoc tests revealed that melatonin values at ZT 15, ZT 18 and ZT 21 were elevated significantly ( $p < .0001$ , respectively) with respect to daytime values. Inferred from ANOVA, the nadir in the body temperature rhythm was between ZT 3 and ZT 6 with significantly elevated temperatures occurring at ZT 15, ZT 18 and ZT 21 ( $p < .001$ ,  $p < .001$  and  $p < .01$ ; respectively) as determined by post hoc comparison.



**Figure 6.1.** Response of equine circadian melatonin and body temperature rhythms to a 6-h phase advance of LD12:12. The LD cycle with 6-h phase advance is depicted



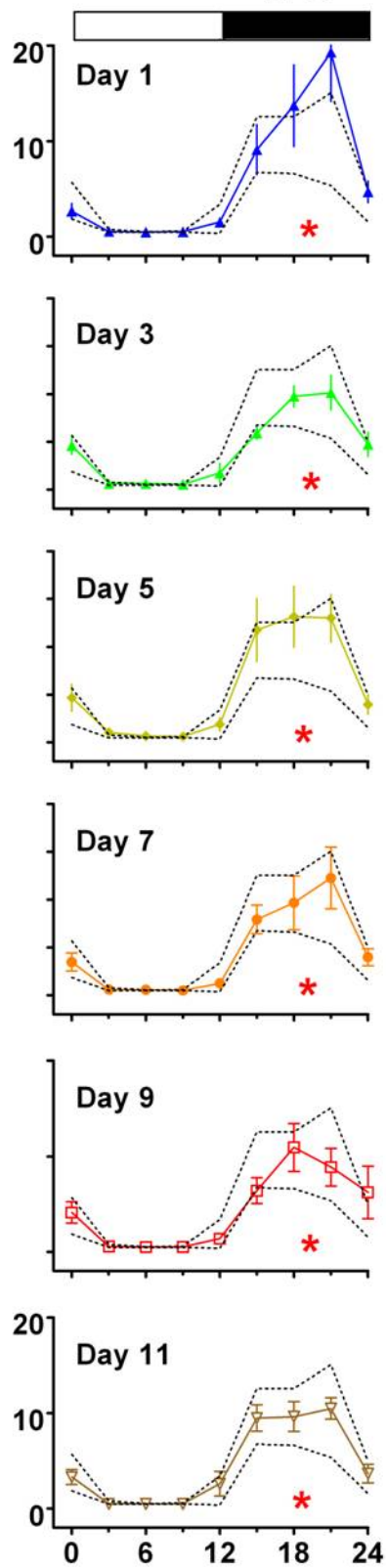
above each graph with white bars representing light and black bars times of darkness. The abscissa represents light cycle time (ZT) in hours, where ZT 0 corresponds to lights on and ZT 12 to lights off of a 12 h photoperiod. Through Day 0 (curves at left) and for one additional day, lights were on 7:30 AM to 7:30 PM (ZT0-12). As indicated by the arrow, the photoperiod was advanced 6 h on Day1 (from ZT18 to ZT 0) to give new lights on from 1:30 AM to 1:30 PM (Days 1-11). Baseline curves (mean $\pm$ SEM) for melatonin (top) and body temperature (bottom) are plotted on the left from ZT 0 to ZT 24. Curves for Day 1 through 11 are plotted on the right with point symbols and SEM removed for clarity. The dotted lines retrace the Day 0 curve assuming no phase shift. Asterisks represent Day 0 mean acrophase times.

#### ***6.4.2 Re-entrainment of melatonin rhythm post phase advance***

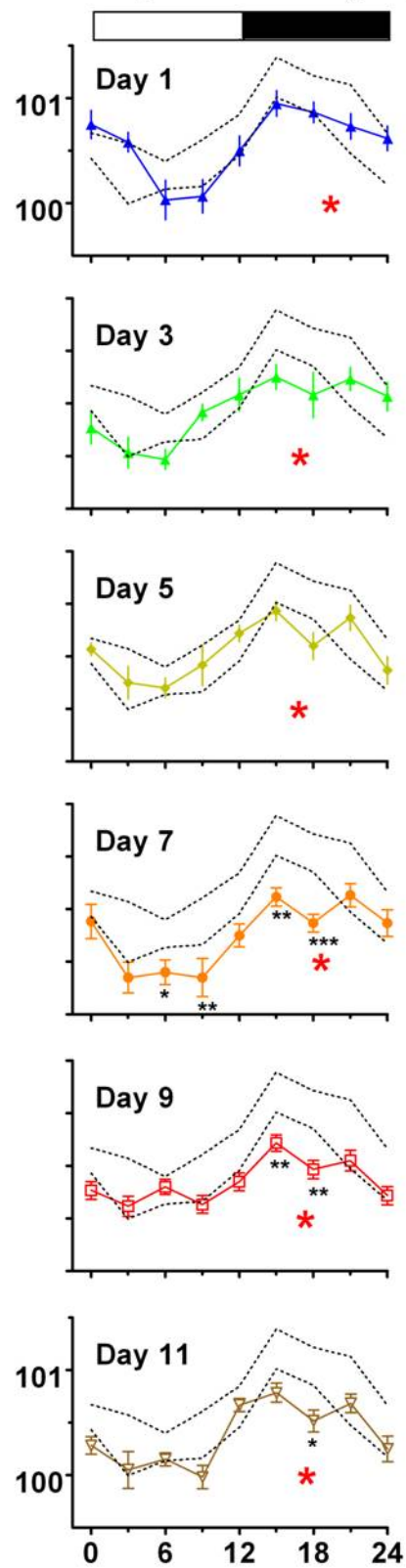
Two-way repeated measures ANOVA of the melatonin data on Day 0 vs Days 1-11 referenced to prevailing ZT times revealed no significant Day X Time interaction on any day post-shift (Figure 6.1; top panel and Figure 6.2A). This is consistent with a full 6-h advance of the rhythm to match the 6 h advance in the ZT scale. Thus, as on Day 0, there was a highly significant ( $p < .0001$ ) effect of time of day on each day (1-11) with elevated levels occurring at the same ZT times as on pre-shift Day 0. Thus by ANOVA referenced to light cycle time (ZT), there was no significant alteration of the melatonin rhythm, thus by inference the rhythm shifted in tune and in time with the LD cycle. The presence of this phase shift is further supported by ANOVA comparison of melatonin data on Day 0 vs Days 1-11 with the time series data referenced instead to unshifted sidereal (real world) clock time. This clock time based ANOVA revealed significant differences in mean melatonin values at 4.30 PM, 7.30 PM, 10.30 AM and 4.30 AM on Day 1 and at 4.30 PM, 7.30 PM, 1.30 AM and 4.30 AM on all other days post-shift. Figure 6.2A illustrates that circadian patterns of serum melatonin concentrations on each day post-shift were normal in waveform and fell within the 95% confidence limits of the phase shifted Day 0 means, with one exception. High melatonin levels were observed at ZT 21 on

Day 1, when three of six mares exhibited abnormally high levels. While precise times of melatonin onset and offset could not be determined from the 3-h sampling protocol, a clear rise in melatonin levels was observed following lights-off (ZT 12) on all days post-shift. Similarly, significantly reduced melatonin values were detected directly following lights-on (ZT 24) on all days post-shift. There was a significant difference in the melatonin acrophase between Day 0 and Days 1-11 ( $p < .0001$ ; Figure 6.3A). The acrophase shifted by five hours on Day 1 and remained stable thereafter. Repeated measures ANOVA revealed that neither the amplitude nor the mesor of the melatonin fitted cosine curves changed significantly on any of the post-shift days (Figure 6.3B).

## Melatonin (pg/ml)



## Temperature (°F)



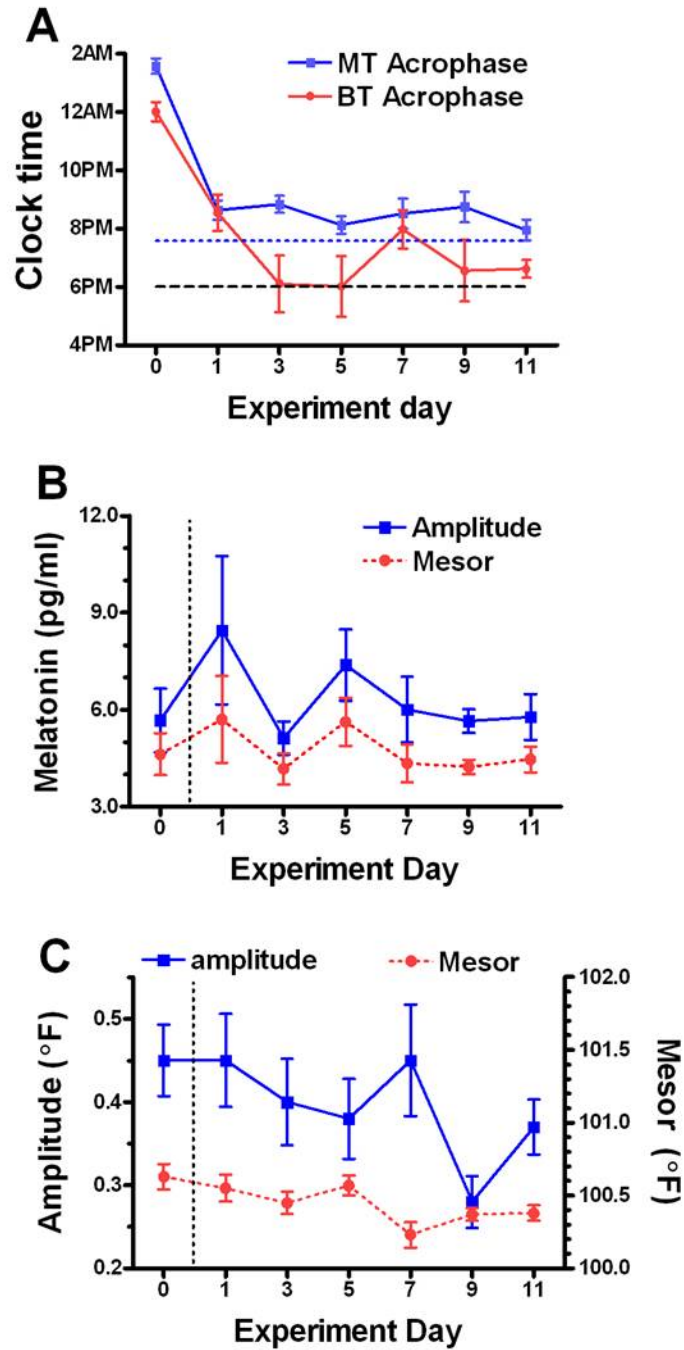
**Figure 6.2.** Time course of reentrainment of melatonin (left) and body temperature (right) rhythms to a 6 h phase advance of LD12:12. The Figure 6.1 curves for Days 1 through Day 11 are re-plotted in a vertical array to show mean $\pm$ SEM in relation to the Day 0 curve, which has been advanced 6h for comparison. Dotted lines connect the upper and lower 95% confidence limits of the phase shifted Day 0 means.

Without exception, Day 1 through Day 11 melatonin curves closely paralleled the zeitgeber time adjusted (6 h advanced) baseline Day 0 curve. By contrast, body temperature curves on Days 7, 9 and 11, deviate significantly from baseline at individual ZT times marked by adjacent black asterisks (\*  $p < .05$ ; \*\*  $p < .01$ ; \*\*\*  $p < .001$ ). Large red asterisks beneath each curve represent cosine fitted acrophases (ZT), which for body temperature, are notably phase delayed in association with the waveform distortions on Days 7-11 (see Figure 6.3A). Other conventions as in Figure 6.1.

#### ***6.4.3 Re-entrainment of body temperature rhythm post phase advance***

Following the 6h phase advance of the LD cycle, there was no significant Day X Time interaction effect on the body temperature rhythm on Day 0 vs. Days 1, 3 and 5, as determined by two-way repeated measures ANOVA (Figure 6.1; bottom panel and Figure 6.2B). On these post-shift days, the shape of the body temperature rhythm was similar to that on pre-shift Day 0, but levels were generally lower and the curves not as sinusoidal as on Day 0. In contrast to the shift in the melatonin rhythm however, there was a significant Day X Time interaction ( $p < .05$ ) with a Day effect manifest in comparing Day 0 vs. Days 7 and 9 ( $p < .001$ ,  $p < .05$ ; respectively). Also, while there was no significant interaction, there was also an effect of Day between Day 0 and Day 11. On these latter post-shift days, post hoc tests revealed significant differences comparing specific time points to the same ZT on Day 0, as denoted (asterisks) on Figure 6.2B. Post-shift disturbances in the waveform of the body temperature rhythm can also be visualized in the number of time points where the

mean temperature falls below the 95 % confidence limits of the phase advanced Day 0 means (Figure 6.2B). While not significant statistically, it is also worth noting the appearance of double peaks (ZT 15 and ZT 21) in the mean body temperature rhythm on all days following Day 1. There was a significant difference in the body temperature acrophase between Day 0 and Days 1-11 ( $p < .0001$ ; Figure 3A). The acrophase shifted by three hours and twenty-eight minutes on Day 1 and completed the 6-h shift by Day 3. While the amplitude of the body temperature rhythm was statistically unaffected by the phase shift, there was a significant effect of experimental Day on the cosine mesor ( $p < .0001$ ), with post hoc differences on Days 1, 5, 7, 9 and 11 (Figure 6.3C), reflecting the overall reduction in mean body temperature post-shift.



**Figure 6.3.** Time course of changes in cosine acrophase, amplitude and mesor for equine melatonin (MT) and body temperature (BT) rhythms with a 6 h phase advance of LD12:12 between Day 0 and Day 1. A. Change in acrophase clock time (mean  $\pm$  SEM) over Days 0 through 11 of the experiment. B. mean  $\pm$  SEM melatonin

amplitude and mesor values  $\pm$  SEM body temperature amplitude and mesor (right y axis). Dotted horizontal lines in A represent a 6 h phase advance relative to baseline (upper, melatonin; lower, body temperature). Note that while the consistent steep rise in melatonin between ZT 12 and 15 on all days suggests a rapid melatonin shift that is essentially complete (+ 6h) on Day 1 and stable thereafter (Figure 6.2A) the daily melatonin acrophases clearly do not lie on the dotted line, while for body temperature they reach the line on days 3 and 5 and then fall back.

**Table 6.1** Melatonin and body temperature mean mesor, amplitude and acrophase values on baseline Day 0 and on Days 1-11 post 6-h phase advance of LD cycle

Melatonin				Temperature		
Day	Mesor (pg/mL)	Amplitude (pg)	Acrophase (CT)	Mesor (° F)	Amplitude (° F)	Acrophase (CT)
0	4.61	5.66	1:35	100.63	0.45	12:01
1	5.7	8.45	8:36	100.55	0.45	8:33
3	4.16	5.13	8:50	100.45	0.4	6:07
5	5.63	7.38	8:08	100.57	0.38	6:00
7	4.35	6	8:31	100.23	0.45	7:58
9	4.23	5.65	8:45	100.37	0.28	6:34
11	4.46	5.78	7:57	100.38	0.36	6:37

## 6.5 Discussion

This is the first experiment of its kind to investigate the re-entrainment of two crucial circadian rhythm phase markers in the horse to an abrupt 6-h advance of the LD cycle. The null hypothesis was that following the 6h phase shift of the LD cycle there would be no change in the phase of the melatonin and body temperature rhythms in relation to the LD cycle. Rejection of this null hypothesis would follow with evidence of acrophase times (ZT) on Days 1 through 11 that differ significantly from the corresponding Day 0 baseline acrophase. As no such differences could be demonstrated, there is, based on cosine analysis, no compelling evidence in the present study for phase-shift related delays in the entrainment of these two equine

rhythms to a 6h phase advance of an LD12:12 cycle. This surprising outcome demonstrating a rapid shift of two markers of circadian phase is discussed further below in the context of human jet lag and in association with a more detailed consideration of the present data.

In humans, jet lag symptoms persist longer following an easterly rather than a westerly flight (Suvanto *et al.* 1990), theoretically, in part because the human clock displays an intrinsic free-running period greater than 24 h, making it more amenable to phase delays than to phase advances (Aschoff *et al.* 1975). In addition, abrupt advances in the LD cycle have recently been shown to result in slower resynchronization of the molecular core clock components of endogenous oscillators within the rat SCN (Nagano *et al.* 2003). This finding suggests that a putative molecular mechanism may underlie the increased severity of physiological symptoms associated with eastward transmeridian air travel. An investigation of the equine circadian body temperature rhythm under constant light (LL) conditions demonstrated a circadian period of 24.2 h (Piccione *et al.* 2002), suggesting that the equine circadian system, similar to that of humans, might have greater difficulty re-entraining to an advance shift of the LD cycle

Our results indicate that in the horse, the melatonin rhythm, often regarded as the best marker of SCN pacemaker phase in humans (Klerman *et al.* 2002), rapidly adjusts to a 6-h shift of the LD cycle. As has been previously observed in almost all mammalian species, including the horse (Guerin *et al.* 1995), the duration of elevated melatonin levels on the baseline Day 0 reflected the duration of the scotophase (dark phase). However, in contrast to human (Barnes *et al.* 1998;Fevre-Montange *et al.* 1981) and animal studies (Drijfhout *et al.* 1997;Illnerova *et al.* 1989;Kennaway 1994) that demonstrate gradual adaptation of the melatonin rhythm to an advanced photoperiod, the equine melatonin rhythm essentially appeared to complete the 6-h phase advance on the first post-shift day, as judged by the acrophase shift and by the 6-h advance in the evening rise of melatonin beginning at ZT 12. Additionally, the lack of distorted waveforms in the days post-shift further supports that stable re-entrainment had been achieved.



It is well known that melatonin production by the pineal gland increases significantly at night in both diurnal and nocturnal species and is a consequence of the high magnitude nocturnal rise in the enzyme serotonin-N-acetyltransferase (Klein *et al.* 1997). The immediate rise in circulating levels of melatonin observed at ZT 12 on Day 0 is not surprising as a similar rapid rise has been observed in sheep immediately following exposure to darkness under conditions of normal 24-h entrainment (Namboodiri *et al.* 1991). However, the observation of a similar rapid rise at ZT 12 on Day 1 post-shift, a time corresponding with the midpoint of the subjective day in the pre-shift photoperiod, stands in stark contrast with what has been observed following phase advances in humans (Fevre-Montange *et al.* 1981; Van Cauter *et al.* 1998). Van Cauter *et al.* (1998) reported only a 2-h advance of the melatonin onset that occurred 6 h after lights-off when human subjects were exposed to the first afternoon dark period following a 6-h LD phase advance. Furthermore, the notable increase in melatonin from baseline levels at ZT 12 on all post-shift days highlights the rapid rise in equine melatonin production within minutes of the new dark period. Similar findings have been reported in the domestic pig (Tast *et al.* 2001). Tast *et al.* (2001) report that an immediate rise in melatonin was observed in response to the first advanced scotophase when pigs were abruptly changed from a long day to a short day LD regimen. The significant reductions in melatonin levels at each sampling time directly following lights-on (ZT 0/ZT 24) supports the well-established ability of light to inhibit melatonin production in humans (Lewy *et al.* 1980).

The rapid response of melatonin to the phase-advanced LD cycle can not distinguish true circadian entrainment from potential masking effects. For example, with the present data we can not know to what extent light suppression as opposed to a fully advanced circadian clock programming the melatonin decline, may have contributed to the precipitous drop in melatonin from ZT 21 to ZT 24 on all days. However, it is less likely that light masking (melatonin suppression) contributed to the abrupt rise in melatonin at ZT 12 – ZT 15 post shift. Importantly, in order to evaluate the physiological disruption caused by time-zone displacement in the natural environment, it is necessary to determine the independent effects that phase shifts exert on circadian rhythms in the presence of an LD cycle.

The unusually high melatonin levels that we observed at ZT 21 on Day 1 of the advanced LD cycle suggest an additive effect whereby the elevated melatonin production in response to the advanced scotophase overlapped with the previously entrained rise (perhaps not fully shifted) in melatonin associated with the pre-shift photoperiod. In the context of multiple SCN oscillators underlying the control of melatonin secretion, the increased secretion at ZT 21 could involve some oscillators advancing more than others to result in an increased overlap of oscillator outputs necessary to drive melatonin secretion occurring transiently at ZT 21. This is perhaps indicative of the combined controlling mechanisms of both the endogenous clock (SCN) and the prevailing LD cycle on melatonin secretion in response to a phase advance, as was similarly suggested for the pig (Tast *et al.* 2001).

In contrast to the rapid and apparently uncomplicated stable re-entrainment of the equine melatonin rhythm, re-entrainment of the body temperature rhythm was slower and not without disturbances in rhythm waveform. The acrophase of the body temperature rhythm was only partially advanced on Day 1 post-shift, requiring three days to completely adjust to the new LD cycle. However, while the acrophase of the fitted cosine curve completed the shift by Day 3, considerably faster than observations of body temperature resynchronization from similar studies in rodents (Goel and Lee 1996; Satoh *et al.* 2006), monkeys (Moore-Ede 1986), and humans (Boivin and James 2002; Wever 1980), the equine body temperature rhythm on subsequent post-shift days displayed significant waveform disturbances. The apparent decline in the robustness of the body temperature rhythm on post-shift days 7, 9 and 11 could suggest acute behavioral masking by the advanced LD cycle, but we know of no data to support this view. Alternatively, transient distortion of the body temperature rhythm post shift may reflect a phase misalignment of multiple SCN, or other, oscillators, which when in normal synchrony contribute to a more robust body temperature rhythm waveform. The double peaks at ZT 15 and ZT 21 which appeared on the latter days post-shift and in all individual rhythms (data not shown), though non-significant in ANOVA, could relate to such desynchrony, i.e. differential advances in body temperature related oscillators in the multi-oscillator SCN (de la Iglesia *et al.* 2004)

Aschoff (1978) first suggested that phase shifts of the LD cycle lead to decreases in amplitude (Aschoff 1978). The lower amplitude of the equine body temperature rhythm on the latter days post-shift may suggest a weakening of the association between the old zeitgeber and the circadian rhythm of body temperature. The finding of less stable rhythms following phase shifts led to the postulation that this weakening helps hasten the shifting of the clock to the new zeitgeber (Aschoff 1978).

The differences in re-entrainment rates between melatonin and body temperature rhythms in the horse likely reflect their independent regulation by different output pathways and different central circadian oscillators perhaps both within and outside the SCN (Moore and Danchenko 2002). Neural connections between the SCN and the paraventricular nucleus of the hypothalamus are thought to regulate melatonin secretion (Klein *et al.* 1983; Larsen *et al.* 1998; Teclemariam-Mesbah *et al.* 1999), whereas the dorsal subparaventricular zone has been identified as the SCN's neural target responsible for the circadian rhythm of body temperature (Lu *et al.* 2001). Our results in the horse appear consistent with the idea that the output mechanism for melatonin may be more directly coupled to the central SCN pacemaker than the output mechanism for body temperature, in this species. This parallels a similar suggestion made with regard to the differential adaptability of body temperature and locomotor rhythms following an LD phase-advance in mice (Satoh *et al.* 2006).

The recent discovery of two spatially distinct oscillators within the SCN (Nagano *et al.* 2003) provides an alternative explanation for the observed differences in re-entrainment rates between melatonin and body temperature. Nagano *et al.* (2003) demonstrate differential resynchronization rates between the ventrolateral, photoreceptive region and the dorsomedial region of the SCN following abrupt LD shifts in rats. Clock gene expression in the ventrolateral region immediately responded to changes in environmental light. In this context, the regulatory mechanisms of the melatonin rhythm might be coupled to the light responsive oscillators within the SCN, thereby potentially explaining the more rapid resynchronization of this rhythm to the new LD cycle in the horse.

The difference between horses and other species in terms of adaptability to abrupt shifts in the LD cycle remains to be fully understood. It is interesting that an animal so dependent on circa-annual cues for seasonal breeding and survival of their young might be more rapidly responsive to circadian re-entrainment. However, we have previously demonstrated that clock gene expression in equine peripheral tissues is less tightly regulated than in other species (Murphy *et al.* 2006). As a large, visible, prey animal in a feral environment, with feeding and rest behaviors unrestricted to specific times in the 24-h natural LD cycle, it is conceivable that the horses' reduced dependency on time cues might also be reflected in a greater capacity for rapid re-entrainment to new environmental conditions.

Jet lag and shift work are considered one of the modern world's most prevalent challenges affecting human health and productivity in the work place. Consequently, extensive resources continue to be employed in the design of potential treatment strategies that might facilitate or hasten re-entrainment and adaptation to the new LD conditions encountered following long flights or shifted work schedules. Our study reveals that in contrast to the human, the horse appears to possess a circadian pacemaker that is more amenable to rapid adjustment to a new photoperiod, suggesting in turn that their performance capacity at a new destination might be less compromised than in human athletes. Further investigation of this phenomenon could potentially provide new insights into re-entrainment mechanisms that might assist in the fight to conquer human jet lag.

## **CHAPTER 7**

### **Conclusions and Future Directions**

#### **7.1 Summary**

When a new path is forged in any field, unexpected findings lead to the formulation of novel hypotheses and often yield results that provoke more new questions than they provide answers. Such is the nature of scientific discovery and is well illustrated in the compilation of studies presented here. An investigation of circadian regulation in the horse was fueled primarily by the desire to determine the physiological impact of phase shifts of the zeitgeber in a species frequently subject to transmeridian travel. The available literature describing the detrimental effects of jet lag on human performance suggested that the potential findings from similar studies in the horse would have important practical implications for the equine industry. Molecular characterization of the equine clockwork mechanism would allow the possibility to examine differential resynchronization of clock gene expression in peripheral tissues, thereby permitting a quantifiable measure of jet lag recovery at a level that encompassed the time delays associated with signaling the phase shift from the SCN to the periphery. Surprisingly, no oscillation of clock gene transcripts was observed in the horse's most accessible tissue, peripheral blood, in contrast to results in other species. These unexpected findings provided the opportunity to test an intriguing hypothesis relating to a potential interaction between the circadian and immune systems that might promote homeostatic recovery during acute systemic inflammation. Importantly, this work brought to light the new scientific insights that can be gleaned from research conducted in a large diurnal mammal such as the horse. In addition, these preliminary investigations of circadian regulation in the horse, both at the molecular and physiological level, provoke many new questions that provide

impetus for continued investigations of equine chronobiology. Suggestions for future research directions will be discussed in more detail below.

To summarize the novel findings from this collection of studies on circadian regulation in the horse; 1) equine clock genes are highly homologous to their human counterparts at both the nucleotide and amino acid level; 2) equine *Bmal1* contains a novel region within the 5'UTR that could potentially exert regulatory control at the translational level; 3) equine clock genes do not exhibit synchronized circadian rhythms of oscillation in peripheral blood in contrast to humans and rodents; 4) systemic inflammation transiently and simultaneously upregulates/synchronizes *Per2* and *Bmal1* expression in peripheral blood; 5) polymorphonuclear neutrophils are the likely source of clock gene upregulation and increased  $\beta$ -glucuronidase activity during acute inflammation in the horse; 6) the equine circadian pacemaker reacts rapidly to a phase advance of the LD cycle.

## **7.2 Novel insertion in equine *Bmal1***

The discovery of a 140 base pair insertion in the 5' UTR of equine *Bmal1* is a finding that is worth further consideration. While 5' UTRs are thought to play a role in post-transcriptional regulation (Bashirullah *et al.* 2001; Jansen 2001), it is unlikely that the unique insertion in equine *Bmal1* disrupts this regulation as the temporal relationships between clock gene oscillations in synchronized equine fibroblasts are similar to the clockwork mechanism described for other species (Lincoln *et al.* 2002; Oishi *et al.* 1998b; Yagita *et al.* 2001). However, it is still possible that this novel insertion exerts translational control. The identification of an upstream ORF suggests that translational efficiency of the main ORF may be impaired due to hampering of the ability of the ribosome to re-initiate at the true start site (Luukkonen *et al.* 1995). This phenomenon is thought to down-regulate translation of mRNA (Vilela *et al.* 1999). A reduced basal translational rate of BMAL1 would impact the positive feedback of CLOCK-BMAL1 heterodimers on *Per* and *Cry* transcription, thus may explain the reduced robustness of clock gene oscillation in equine adipose tissue and contribute to the lack of oscillation in equine peripheral blood. Future work

could include sequence analysis to test for the presence of this insertion in members of the perissodactyl lineage representing all three families (Tapiridae, Equidae, and Rhinocerotidae). The availability of genomic DNA would allow PCR analysis of the area surrounding the insertion. This would permit investigation of when in evolutionary time the variation emerged and of how well it has been conserved as the species diverged. Furthermore, continued sequencing of the full length cDNAs for the other core clock genes might reveal additional genetic variations that would further aid in understanding circadian regulation in the horse.

### **7.3 Inflammation, clocks and apoptotic neutrophils – implications for cancer treatment?**

The studies presented here describe a previously unknown interaction between the circadian and immune systems of the horse in response to LPS. Synchronized upregulation of *Bmal1* and *Per2* at the time corresponding with both the peak in fever and significantly increased levels of circulating neutrophils suggests that the circadian system has a role to play in restoring homeostasis during the innate immune response. These results spurred a subsequent ex vivo investigation that demonstrated that the febrile mediator, PGE<sub>2</sub>, is sufficient to mediate this effect in cultured equine neutrophils. While the ability of the NSAID, phenylbutazone, to inhibit the clock gene rise in vivo supports this finding, it is not possible to conclude that PGE<sub>2</sub> is necessary for the response, as other mediators involved in this interaction may also have been blocked by the drug. However, previous studies demonstrating the phase resetting capability of this hormone in mouse peripheral tissues further supports a regulatory role for PGE<sub>2</sub> on clock gene expression in equine blood. One likely function of clock gene involvement in inflammation relates to the new role of BMAL1 in augmenting histone acetyl-transferase activity (Doi *et al.* 2006) thereby permitting the large scale transcriptional changes associated with immune cell activation (Fannin *et al.* 2005; Malcolm *et al.* 2003).

The finding of an enhanced clock gene transcriptional response to PGE<sub>2</sub> associated with reduced neutrophil viability provides an additional opportunity to learn more about the potential interactions between inflammation, neutrophils and the molecular clock. Ex vivo induction of neutrophil apoptosis and assessment of cell status using Annexin V staining, that allows detection of the early stages of apoptosis, would provide a more ideal method of determining the effect of PGE<sub>2</sub> and LPS on transcriptional responses in apoptotic cells. An important application for such an experiment would be to identify the source and mediator of the increased  $\beta$ -glucuronidase activity observed in inflammatory conditions (Marshall *et al.* 1988) and in cancerous tissue (Bosslet *et al.* 1998). Previously, it has been shown that this hydrolyzing enzyme, responsible for deglucuronidation of inactive glucuronides to active parent compounds, is released from stimulated neutrophils and injured cells (Shimoi and Nakayama 2005). However, it was unknown whether transcription of *GUS* was induced in these conditions. Our preliminary results strongly suggest that PGE<sub>2</sub> mediates significantly increased upregulation of *GUS* transcripts in less viable neutrophils. These results could be confirmed definitively using the above proposed experimental protocol and would likely serve to identify one of the mediators responsible for the high  $\beta$ -glucuronidase activity observed in inflammatory tissues. This is especially important since one strategy to overcome the adverse effects associated with high dose anticancer agents, necessary for sufficient drug concentration in tumor tissue, is administration of nontoxic glucuronide prodrugs from which the active moiety is released by  $\beta$ -glucuronidase activity within or near the tumor (Murdter *et al.* 1997; Sperker *et al.* 2000). However, the use of glucuronide prodrugs in cancer treatment requires increased expression of the enzyme in the diseased tissue, a problem that is confounded by interindividual variability in expression of *GUS* (Sperker *et al.* 1997). Therefore, the identification of an inflammatory mediator capable of significantly upregulating *GUS* expression in apoptotic neutrophils could potentially provide crucial new information that would benefit future anti-cancer drug strategies. In addition, investigating the temporal relationships between upregulation of clock genes and *GUS* in apoptotic cells in



response to PGE<sub>2</sub> would shed light on the role of the molecular clock in this phenomenon.

#### **7.4 Do horses suffer from jet lag?**

The overall goal of this research at the outset was to determine the duration of physical misalignment associated with an abrupt phase advance of the photoschedule in the horse. The results of the subsequent phase shift experiment demonstrate extraordinarily rapid re-entrainment of melatonin and core body temperature rhythms, in stark contrast to the slow re-entrainment rates observed in multiple other species. These discoveries necessitate reformulation of the original question. As melatonin and body temperature are physiological outputs that have been used predominantly as phase markers that represent the ticking of the internal mammalian clock, the rapid readjustment of these markers in the horse could be interpreted as the absence of the jet lag phenomenon in this species. However, before hastily jumping to this conclusion it is necessary to examine the evidence more closely. While there is an absence of waveform disturbances in the shifted circadian rhythm of serum melatonin, the appearance of two peaks in the body temperature rhythm in the latter days post-shift point to a separation of dual regulatory inputs to this behavioral clock output. The question remains however whether this slight body temperature rhythm anomaly would translate into performance deficits or truly represents disturbances at the pacemaker level. Considering that the normal body temperature rhythm in the horse peaks during early night, which is in itself unusual for a diurnal animal, coupled with the tendency to exercise horses in the early morning hours, it is unlikely that the observed variations in the body temperature rhythm would negatively impact performance at these times. An interesting and worthwhile future endeavor would be to compare race times, as a marker of performance capacity, at the same track during both morning and evening meets to determine if there is a diurnal preference for improved performance closer to the peak in body temperature.

The immediate response of melatonin secretion at the time of the first shifted dark period is an interesting observation. As mentioned previously, a similar

experiment using human subjects found that the onset of melatonin only occurred 6 h after lights-off (Van Cauter *et al.* 1998). The apparent immediate onset of equine melatonin production to darkness is similar to observations in pigs (Tast *et al.* 2001). Two important experiments would further characterize this phenomenon in the horse. First, transferring animals from an LD cycle into constant dim light would allow the free-running melatonin rhythm to be recorded and would demonstrate that the prevailing LD cycle is not the sole driving force behind this rhythm. Second, exposing horses transferred from LD to constant light to short dark pulses at intervals throughout the 24-h cycle would determine whether the ability of darkness to drive melatonin production varies over time.

The recent elucidation of the molecular mechanism responsible for the feedback effect of melatonin on the SCN reveals the involvement of the nuclear orphan receptor *Rev-erba* (Agez *et al.* 2007). This highlights a direct link between a clock output (the rhythm of melatonin synthesis) and synchronization of the molecular clockwork mechanism. Nuclear orphan receptors act as functional links between the regulatory loops of the molecular clock and specifically, *RORα* and *Rev-erba* regulate *Bmal1* transcription (Guillaumond *et al.* 2005). It is now conceivable that the physical malaise and performance deficits associated with LD phase shifts in humans might be partially attributed to the slow resynchronization of the melatonin rhythm and consequently, a slower resetting of the phase of the molecular clock in peripheral tissues where melatonin receptors are widespread (Naji *et al.* 2004; Pang *et al.* 1993). On the same note, the rapid advancement of the equine melatonin rhythm to a new LD cycle might reset molecular clocks in peripheral tissues, thus significantly reducing the negative impact on physiological rhythms suggested to arise from molecular desynchronization (Reddy *et al.* 2002). While still purely speculative, future investigations of circadian gene expression, clock gene rhythmicity and re-entrainment times in alternative equine peripheral tissues, such as muscle and liver, would provide concrete evidence to support or refute the ability of horses to rapidly adapt to a new time-zone at all levels of physiology. The new microarray technology that will become available to equine scientists in the wake of the equine genome project ([www.uky.edu/ag/horsemap](http://www.uky.edu/ag/horsemap)) will permit evaluation of the

subsets of genes under circadian control in specific tissues. Thus, future experiments could evaluate entrainment times of genes specific to performance capacity (Rankinen *et al.* 2006).

The potential physiological capability of the horse to elude the effects of jet lag should generate significant interest within a society that employs extensive resources in the design of potential drugs and treatment strategies that might facilitate, or hasten, re-entrainment of circadian rhythms in cases of jet lag and shift work (Burgess *et al.* 2003; Cardinali *et al.* 2002; Eastman *et al.* 2005; Parry 2002). As an interesting concluding example, the most recent drug tested to phase advance hamsters prior to a 6-h LD shift, sildenafil, is more commonly recognized as a human treatment for erectile dysfunction (Agostino *et al.* 2007). The drug clearly reduces the duration of circadian desynchrony following an abrupt phase advance in hamsters, which serves to highlight the fact that when used for its prescribed function, it also likely disrupts normal circadian entrainment. Hence, this may be the newest example of a societal advance with consequences for circadian rhythmicity.

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## VITA

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#### **Education**

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| 2001-present | University of Kentucky, College of Agriculture,<br>Department of Veterinary Science. Doctoral candidate<br><b>GPA 4.0</b>                           |
| 1996-2000    | University of Limerick, College of Life Sciences,<br>Limerick, Ireland.<br>Awarded Bachelor of Science in Equine Science with<br>First Class Honors |

#### **Awards and Honors**

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- University of Kentucky Graduate School Travel Support Funding 2007
- Research Activity Award in support of innovation and excellence from the College of Agriculture, University of Kentucky 2005-2006
- Neal A. Jorgenson Equine Genome Travel Award 2006
- Research Assistantship, Department of Veterinary Science, University of Kentucky 2001- present
- Gamma Sigma Delta Agriculture Honor Society Member 2003 - present
- Ford Prize for Excellence in Equine Science, University of Limerick 2000

#### **Association Memberships**

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- Society for Neuroscience 2005- present
- Society for Research on Biological Rhythms 2003- present
- American Society of Animal Science 2003-2004

## Publications

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**Murphy BA**, Elliott JA, Vick MM, Sessions DR, Kennedy EL, Fitzgerald BP (2007) Rapid phase adjustment of melatonin and core body temperature rhythms following an abrupt 6 h phase advance of the light/dark cycle in the horse. *Journal of Circadian Rhythms*. 5:5 doi:10.1186/1740-3391-5-5.

**Murphy BA**, Lear TL, Adelson DL, Fitzgerald BP (2007) Chromosomal assignments and sequences for the equine core circadian clock genes. *Animal Genetics*. 38: 84-85

**Murphy BA**, Vick MM, Sessions DR, Cook RF, Fitzgerald BP (2007) Acute systemic inflammation transiently synchronizes clock gene expression in equine peripheral blood. *Brain, Behavior and Immunity*. 21: 467-476

**Murphy BA**, Vick MM, Sessions DR, Cook RF, Fitzgerald BP (2006) Evidence of an oscillating peripheral clock in an equine fibroblast cell line and adipose tissue but not in peripheral blood *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* 192: 743-751

Vick MM, **Murphy BA**, Sessions DR, Reedy SE, Kennedy EL, Horohov DW, Cook RF, Fitzgerald BP. (2007) Systemic inflammation induces insulin resistance in the horse and increases inflammatory cytokine expression in equine adipose tissue. *American Journal of Veterinary Research*. In press.

Vick MM, Adams AA, **Murphy BA**, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP (2007) Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *Journal of Animal Science*. 85:1144-55.

Vick MM, Sessions DR, **Murphy BA**, Kennedy EL, Reedy SE, Fitzgerald BP (2006) Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. *Reproduction, Fertility and Development* 18 (6): 609-616.

Sessions DR, Reedy SE, Vick MM, **Murphy BA**, Fitzgerald BP (2004) Development of a model for inducing transient insulin resistance in the mare: preliminary implications regarding the estrous cycle. *Journal of Animal Science*. 82: 2321-2328.

In preparation: **Murphy BA**, Mérand C, Vick MM, Cook RF, Horohov DW, Fitzgerald BP. Equine neutrophils respond to PGE<sub>2</sub> by activating expression of core circadian clock genes.

## **Presentations**

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Rapid resynchronization of melatonin and core body temperature rhythms following a 6 h phase advance of the light/dark cycle in the horse. Gordon Research Conference on Chronobiology. 2007, Aussois, France. Poster presenter.

Equine chronobiology: The story so far... Seminar to members of the human and hamster circadian rhythm research group at the Department of Psychology, University of California, San Diego (UCSD). January 18<sup>th</sup>, 2007. Invited speaker.

Clock gene expression in equine peripheral tissues. Seminar to the Department of Veterinary Science, University of Kentucky. November, 2005.

A functional genomics approach to investigating the biological clock in the horse. 6<sup>th</sup> International Equine Gene Mapping Workshop held in Kildare, Ireland. July, 2005. Invited speaker.

Clock genes in the horse: Relevance for investigation and preliminary results. Oral Presentation to the Circadian Rhythm Journal Club at the Chandler Medical Center, University of Kentucky. April, 2005..

Equine Chronobiology. Seminar to the Department of Veterinary Science, University of Kentucky. December, 2004.

## **Abstracts**

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Brooks SA, **Murphy BA**, Bailey E (2007) Changes in expression of mast cell related genes in response to IV LPS: Preliminary data. Proc. 7<sup>th</sup> International Equine Genome Mapping Workshop. Tahoe City, CA.

**Murphy BA**, Vick MM, Sessions DR, Cook RF, Fitzgerald BP (2007) Molecular evidence of immune-circadian interaction in the horse. Program Number P602. San Diego, CA: Plant & Animal Genomes XV Conference, 2007.

**Murphy BA**, Vick MM, Sessions DR, Cook RF, Fitzgerald BP (2006) Acute systemic inflammation induces upregulation of circadian clock genes *Per2* and *Bmal1* in equine peripheral blood. Proc Soc Res Biol Rhythms Tenth Mtg Abstr 221. Destin, FL.

**Murphy BA**, Vick MM, Sessions DR, Cook RF, Fitzgerald BP (2006) Investigation of the equine molecular clock in a fibroblastic cell line, adipose tissue and peripheral blood. Program Number P600. San Diego, CA: Plant & Animal Genomes XIV Conference, 2006. .



Bellone RR, Archer S, **Murphy BA**, Bailey, E. Evidence for TRPM1 as the genetic cause of Appaloosa spotting (LP) (2007). Program Number P597. San Diego, CA: Plant & Animal Genomes XV Conference, 2007.

Brooks SA, Lear TL, Adelson D, **Murphy BA**, Bailey E (2006) Characterization of a paracentric inversion on ECA3 associated with the tobiano spotting pattern in horses. Program Number P597. San Diego, CA: Plant & Animal Genomes XIV Conference, 2006.

**Murphy BA**, Cook RF, Vick MM, Sessions DR, Fitzgerald BP (2005) Oscillating expression of core clock genes in the horse Program No. 60.15. 2005 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2005.

**Murphy BA**, Lear TL, Brooks SA, Adelson D, Fitzgerald BP (2005) A functional genomics approach to investigating the biological clock in the horse. Proc 6<sup>th</sup> International Equine Gene Mapping Workshop. Kildare, Ireland.

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